ISSN (Online): 2455-7838
SJIF Impact Factor (2016): 4.144

EPRA International Journal of

Research & Development

(IJRD)

Monthly Peer Reviewed & Indexed International Online Journal

Volume:2, Issue:1, January 2017

Published By :
EPRA Journals

SJIF Impact Factor: 4.144 ISSN: 2455-7838(Online)
EPRA International Journal of Research and Development (IJRD)
Volume: 2 | Issue: 1 | January | 2017

MASS SPECTROMETRIC ANALYSIS OF 2 DE PROTEINS ISOLATED FROM BISPHENOL A INDUCED AND PROBIOTIC TREATED HYPOTHALAMUS AND LIVER TISSUES OF SPRAGUE DAWLEY RATS

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ABSTRACT

Bisphenol A (2, 2-bis (4-hydroxyphenyl propane) (BPA)is an endocrine disruptor compound and exposure to high doses of BPA causes toxicity in multiple organ systems such as the kidney, liver, spleen and pancreas. In the present study, we were isolated the hypothalamus and liver tissues of healthy Rattus norvergicus, treated with BPA and BPA with probiotic bacteria Lactobacillus salivarius for 14 days. The tissues were isolated and carried out for 2DE analysis. In the 2 DE, we have identified some over expressed proteins in Bishenol treated liver and hypothalamus tissue. Under probiotic treatement, the expression of proteins lower than the Bisphenol treated and higher than the control levels. This is because of both transcription and translation processes under stress These proteins are undergone for MSMS analysis and the proteins identified and grouped into four categories: ***1)*** *Bisphenol induced proteins in Hypothalamus are gamma-actin partial [Musmusculus], Protein transport protein Sec31A, Atp5b protein [Musmusculus].* ***2)*** *Bisphenol and probiotic induced proteins in Hypothalamus are polyadenylate-binding protein 4 isoform X3, V-type proton ATPase catalytic subunit A.* ***3)*** *Bisphenol induced proteins in Liver are INSL3_MOUSE Insulin-like 3, Mitochondrial ATP synthase H+ transporting F1 complex beta subunit, Laminin subunit alpha-5 precursor* ***4)*** *Bisphenol with probiotic induced proteins in liver are Nucleolar protein 14, Nuclear protein MDM1. These results show the value of proteomic approach in identifying the differentially expressed proteins in cellular homeostasis influenced by Bisphenol A.*

KEYWORDS: *Bisphenol A(BPA), Rat hypothalamus and liver, Blood reports, Histopathology,2D electrophoresis, Proteomics, MS/MS ANALYSIS.*

1. INTRODUCTION

Proteomics can provide information about the conditions under which a protein might be expressed, its cellular location, the relative quantities and what protein–protein interactions take place. Although new methods using multidimensional liquid chromatography to identify proteins in mixtures have appeared, protein separation by 2DE and subsequent protein identification by mass spectrometry is a widely used strategy in proteomics [1],[2]. The utility of mass spectrometry for protein and peptide analysis lies in its ability to provide highly accurate molecular weight information and characterization. In fact, proteomics has known a strong evolution and now we are in a phase of unparalleled growth that is reflected by the amount of data generated from each experiment. Bioinformatics applied to proteomics offered the management, data elaboration and integration of these huge amount of data. Mascot is a software search engine that uses mass spectrometry data to identify proteins from peptide sequence databases [3].

2. SAMPLING DESIGN

Protein Extraction from hypothalamus and liver Tissues:

1gm of both the hypothalamus and liver tissue were weighed and grinded in 50mM Tris Buffer. Homogenization of the samples were carried out and the samples were centrifuged at 14000rpm.The supernatants obtained in both the individual tissues were collected into separate tubes.

2 DE:

The samples were loaded on to the IEF strips 3-10pH Linear, 18cm and kept for Iso-Electric Focusing. After IEF run, the strip was equilibrated in Equilibration Buffer and the second dimension was carried out on a 10% SDS-PAGE. The gels were Silver stained to observe the protein spots and were scanned using Epson Expression 11000XL Scanner. The image analysis of the gels was carried out using Image Master 2D Platinum 7.

3. METHODOLOGY

MS/MS Analysis:-

The gel slice was diced to small pieces, placed in new eppendorff tubes and gel pieces were destained using destaining solution for 10minute intervals (3-4 times) until the gel pieces become translucent white. Then gels were dehydrated using acetonitrile and Speedvac till complete dryness. These gel pieces were rehydrated with DTT and incubated for an hour. After incubation, the DTT solution was removed. The gel pieces were now incubated with Iodoacetamide for 45min.The supernatant was removed and the gel was incubated with ammonium bicarbonate solution for 10min.The supernatant was removed and the gel was dehydrated with acetonitrile for 10min and Speedvac till complete dryness. Trypsin solution was added and incubated overnight at 37^0C.The digest solution was transferred to fresh eppendorff tubes. The gel pieces were extracted thrice with extraction buffer and the supernatant was collected each time into the eppendorff above and then Speedvac till complete dryness. The dried pepmix was suspended in TA buffer. The peptides obtained were mixed with HCCA matrix in 1:1 ratio and the resulting 2ul was spotted onto the MALDI plate. After air drying the sample, it was analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument and further analysis was done with FLEX ANALYSIS SOFTWARE for obtaining the PEPTIDE MASS FINGERPRINT. The masses obtained in the peptide mass fingerprint were submitted for Mascot search in "CONCERNED" database for identification of the protein.

Protein identification:-

PMF data was investigated for protein identification with NCBI database for Rattus norvergicus using Mascot search engine and analysis was done on global proteomic solutions (GPS) software automatically. Database searches were carried out are summarized in the following Table

Table: 1 Mascot search parameter

Parameter	Value
Taxonomy	Rattus norvergicus
Database	Swissprot
Maximum missed cleavage	1
Fixed modification	Carbamido methyl (c)
Variable modification	Oxidation
Enzyme	Trypsin
Peptide tolerance	200-1200ppm
Peptide charge	+1
Data format	Mascot generic
MS tolerance	0.2-2 Da

4. RESULTS

I List of proteins expressed from Bisphenol A induced and Probiotic treated Hypothalamus of Rattus norvegicus.

A) **BH3- gamma-actin, partial [Musmusculus] protein:**The protein interacts selectively and non-covalently with ATP, adenosine 5'-triphosphate, a universally important coenzyme and enzyme regulator. The other biological functions include platelet aggregation (The adhesion of one platelet to one or more other platelets via adhesion molecules and retina homeostasis(4) A tissue homeostatic process involved in the maintenance of an internal equilibrium within the retina of the eye, including control of cellular proliferation and death and control of metabolic function (5)

B) **BH4- Protein transport protein Sec31A:**This protein is the component of the coat protein complex II (COP II) which promotes the formation of transport vesicles from the endoplasmic reticulum(ER).The coat has two main functions, the physical deformation of the endoplasmic reticulum membrane into vesicles and the selection of cargo molecules. COP II is composed of atleast 5 proteins: the SEC 23/24 complex, the SEC 13/31 complex and SAR1. Interacts with PDC6 in a calcium dependent manner (6)

C) **BH5- Atp5b protein [Musmusculus]** :Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F_1 - containing the extramembraneous catalytic core, and F_0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F_1. Rotation of the central stalk against the surrounding $alpha_3beta_3$ subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits. Catalytic activity ATP + H_2O + H^+(In) = ADP + phosphate + H^+(Out) (7)

D) **BPH3-PREDICTED: polyadenylate-binding protein 4 isoform X3:** Interacts selectively and non-covalently with a sequence of adenylyl residues in an RNA molecule, such as the poly(A) tail, a sequence of adenylyl residues at the 3' end of eukaryotic mRNA. myeloid cell development and regulation of mRNA stabilty are the biological process of this protein .

E)BPH4- V-type proton ATPase catalytic subunit A:The function of this protein is catalytic subunit of the peripheral V1 complex of vacuolar ATPase. Vacuolar ATP ase is responsible for acidifying a variety of intracellular compartments in eukaryotic cells.

II List of proteins expressed from Bisphenol A and Probiotic treated liver tissues of Rattus norvegicus

A) BL1384-INSL3_MOUSE, Insulin-like 3: It Seems to play a role in testicular function. It may be a trophic hormone with a role in testicular descent in fetal life. This protein is a ligand for LGR8 receptor. The location is subcellular and it is secreted Subunit structure. Heterodimer of a B chain and an A chain linked by two disulfide bonds. The Molecular function is protease binding. Biological process involves in utero embryonic development, male gonad development, positive regulation of epithelial cell migration, positive regulation of wound healing, regulation of male gonad development(8).

B) BL 1500- Mitochondrial ATP synthase, H+ transporting F1 complex beta subunit

Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F_1 - containing the extra membraneous catalytic core, and F_0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits of alpha and beta form the catalytic core in F_1. Rotation of the central stalk against the surrounding $alpha_3$ $beta_3$ subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits.

Catalytic activity :ATP + H_2O + H^+(In) = ADP + phosphate + H^+(Out).

C) BL1549- Laminin subunit alpha-5 precursor

Its function is Binding to cells via a high affinity receptor, laminin is thought to mediate the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components. Laminin is a complex glycoprotein, consisting of three different polypeptide chains

(alpha, beta, gamma), which are bound to each other by disulfide bonds into a cross-shaped molecule comprising one long and three short arms with globules at each end. Alpha-5 is a subunit of laminin-10 (laminin-511), laminin-11 (laminin-521) and laminin-15 (laminin-523). Alpha-5 may be the major laminin alpha chain of adult epithelial and/or endothelial basal laminae.

D)BPL 119- Nucleolar protein 14

This protein is involved in nucleolar processing of pre-18S ribosomal RNA. It has a role in the nuclear export of 40s pre-ribosomal subunit to the cytoplasm.

E)BPL 128-Nuclear protein MDM1

The subcellular location is nucleus. This protein is a microtubule-binding protein that negatively regulates centriole duplication, binds to and stabilizes microtubules. Widely expressed at high levels in testis.

5. CONCLUSION

The present study have established the proteomic analysis of Bisphenol A stressed, BPA and Probiotic treated proteins by mass spectrometry technique. The spectrum was measured for unknown peptides and were compared against the mass peaks derived from calibration of internal standards. BPA induced hypothalamus proteins involved in platelet aggregation, vesicular protein transport and ATP synthesis.where probiotic treated proteins shown regulating mRNA stability and ATP ase activity counter to that of ATP synthase.Maximum spectrum was observed in Bisphenol stressed liver protein spots. Testicular function, ATP production and, cell adhesion are the areas of functions of this proteins where the BPA with probiotic treated tissue proteins are involved in nucleolar processing and centriole duplication. This seems role of probiotics in negatively regulating the translation and over cell duplication process, there by bisphenol alters the different metabolic functions. Further combining the proteomics technology with bioinformatics might lead to identification of complete protein information of analysed proteins.

Table: 2
Haemotological studies of Rattus Norvergicus induced with Bisphenol A with Bisphenol A and probiotic bacteria

S.No	Name of test	Test Samples			Results
	Renal function test	**control**	**Bisphenol A induced blood**	**BPA+probiotic Treated blood**	**in units**
1.	Serum creatinine	1.3	0.8	0.9	mg/dl
2.	Serum uric acid	56.3	24.2	33.3	mg/dl
3.	Blood urea	0.00	191.3	36.4	Mg/dl

S.No	Name of test	Test Samples			Results
	Complete blood picture (CPV)	**control**	**Bisphenol A induced blood**	**BPA+probiotic Treated blood**	**in units**
4.	Haemoglobin	7.7	6.5	9.7	g/dl
5.	RBC	0.07	0.32	0.20	millions/cum
6.	Platelet count	59,000	6.94	2.2	Lcks/cumm
7.	PCV	0.4	1.8	1.5	%
8.	HbSAg	NEGATIVE	NEGATIVE	NEGATIVE	--
9.	HIV I &II TRI-DOT	Non-reactive	Non-reactive	Non-reactive	--
10.	Blood Group&Rh typing	"A"positive	"A"positive	"A"positive	--

Table: 3
List of proteins expressed from Bisphenol A induced and Probiotic treated Hypothalamus of Rattus norvegicus.

S.No	Spot no	Protein name	Molecular weight	Calculated pI	Number of amino acids
1.	BH3	gamma-actin, partial [Musmusculus]	41018.9	5.56	268
2.	BH4	Protein transport protein Sec31A	133569.2	6.30	1230
3.	BH5	Atp5b protein [Musmusculus]	56666.8	5.24	533
4.	BPH3	PREDICTED: polyadenylate-binding protein 4 isoform X3	69331.3	9.58	630
5.	BPH4	V-type proton ATPase catalytic subunit A	68326.0	5.41	617

APPENDIX

Table :4
List of proteins expressed from Bisphenol A and Probiotic treated liver tissues of Rattus norvegicus

S.No	Spot no	Protein name	Molecular weight	Calculated pI	Number of Amino acids
1.	BL1384	INSL3_MOUSE, Insulin-like 3	13585.8	9.25	122
2.	BL1500	ATP synthase subunit beta, mitochondrial precursor [Musmusculus]	56300.4	5.19	529
3.	BL1549	Laminin subunit alpha-5 precursor	404053.6	6.28	3718
4.	BPL119	Nucleolar protein 14	98769.4	7.34	860
5.	BPL128	Nuclear protein MDM1	75673.7	9.33	673

Figure 1: Histopathology of Hypothalamus tissue of *RATTUS NORVERGICUS*

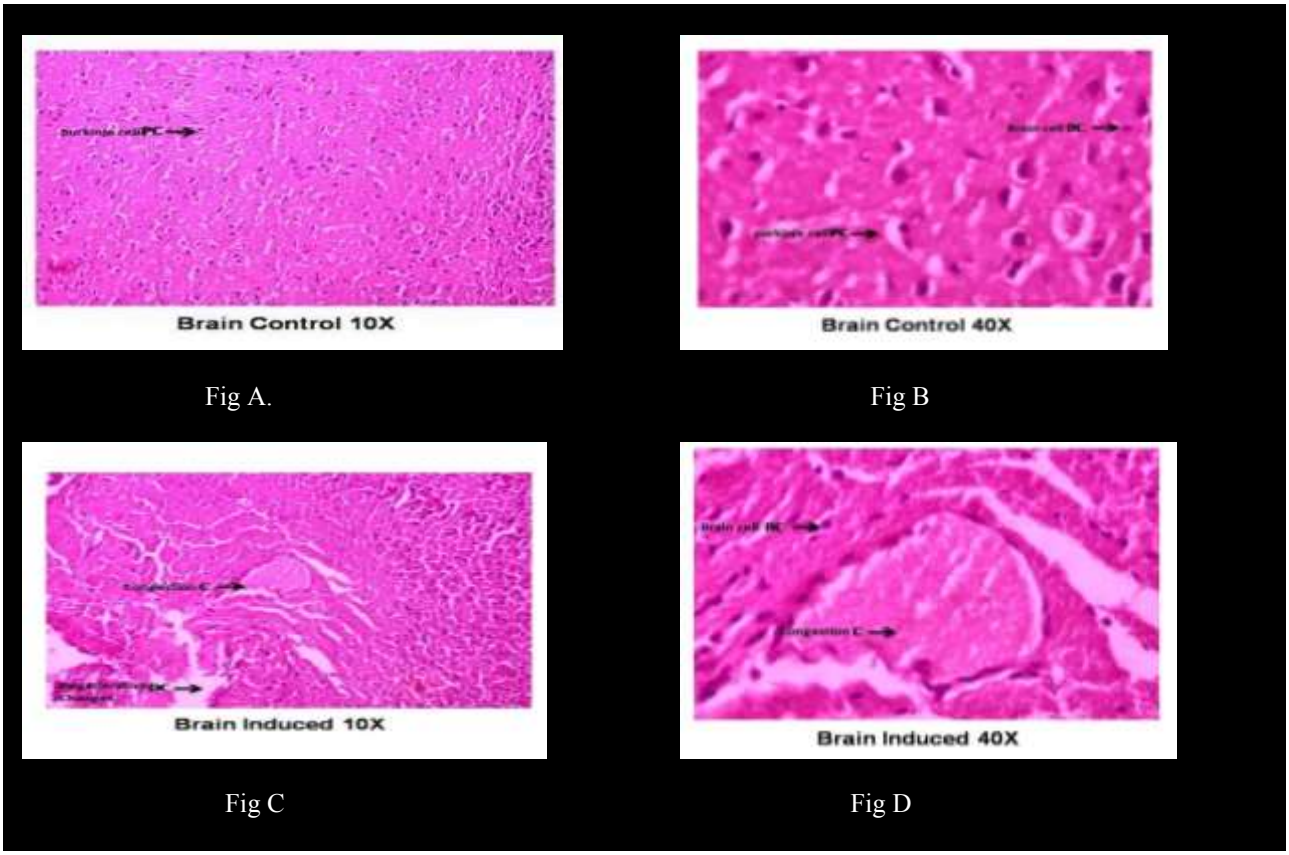

Fig A.

Fig B

Fig C

Fig D

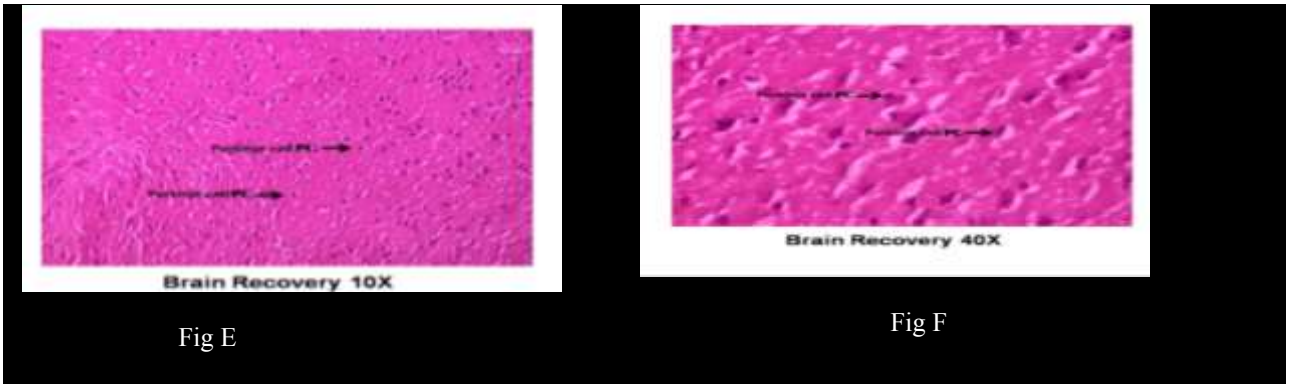

Fig E

Fig F

Fig A&B are photomicrograhs for Hypothalamus histological changes in control rat shows purkinje cells (PC) under 10X. A higher magnification showing both brain cell (BC) and purkinje cell(PC). Fig C&D photomicrographs of hypothalamus section of rat induced with BPA shows congestion(C) an Degenerative changes (DC), under 40X congestion is clearly visible . Fig E&F are photo micrograph section of rat hypothalamus recovered from BPA shows purkinje cells(PC).

Figure:2 Histopathology of Liver tissue of *RATTUS NORVERGICUS*

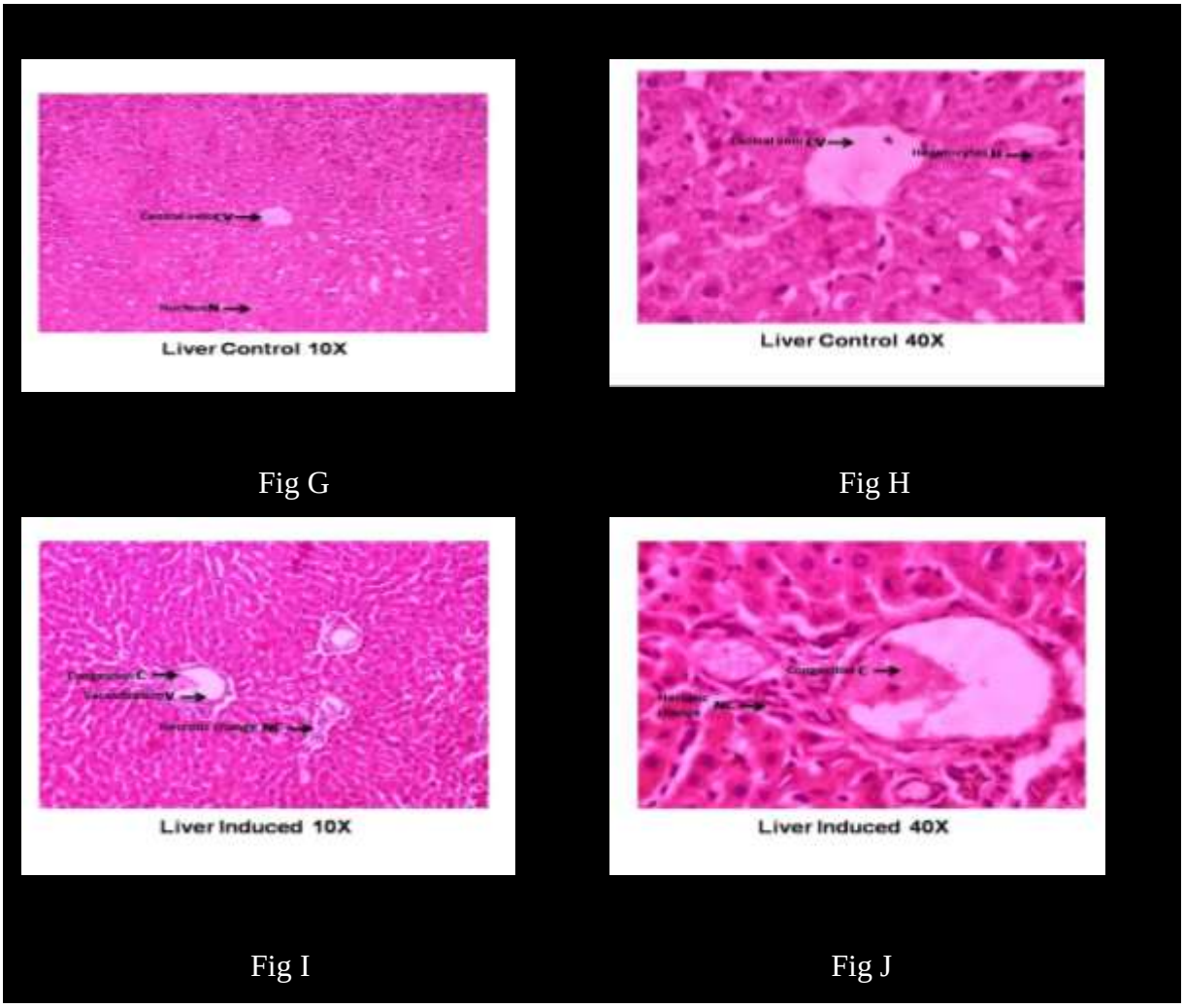

Fig G

Fig H

Fig I

Fig J

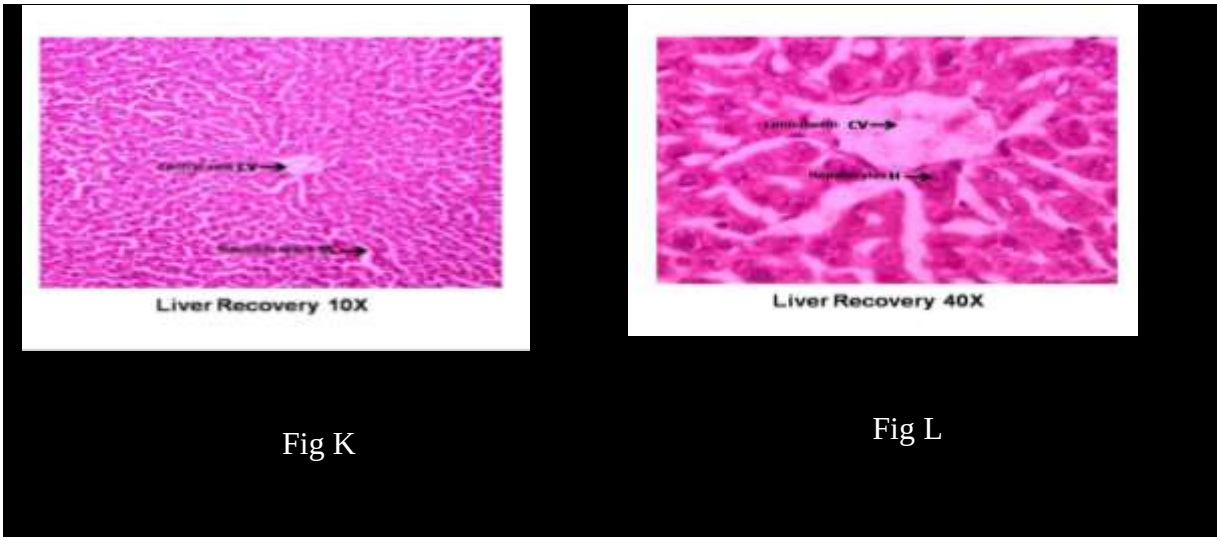

Fig K

Fig L

Fig G&H are photomicrographs of liver sections of control rat showing normal hepatic plates radiating from a thin walled central vein (CV) and Nucleus (N)under 10X lens and hepatocytes contained rounded vesicular nuclei seen under 40X lens. Fig I&J are photomicrographs of BPA induced rat liver showing presence of Congestion, vacoulisation and Necrotic change. FigK&L are photo micrographs of BPA induced sections of rat liver that are covered by probiotic bacteria showing presence Central vein (CV) ,sinusoids space (SS) under 10x Central vein (CV) Hepatocytes (H) under 40 X.

ACKNOWLEDGEMENT

The authors are thankful to UGC, New Delhi for providing financial assistance (UGC-MRP).

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