Histopathological effects of Nicotine on the liver of Indian major carp, *Labeo rohita* (Hamilton, 1822).

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ABSTRACT

Endocrine Disrupting Compounds (EDCs) are produced naturally and commercially through various anthropogenic activities. Both of these types are capable of producing a certain endocrine disruption in the physiology of organisms dwelling in that environment. A multitude of study revealed and recorded the occurrence of Endocrine Disrupting Compounds in Indian freshwater which were at potentially toxic levels. One such commercial compound was Nicotine, known to be a potential endocrine disruptor since studies have revealed it capable of altering estrogen responsive enzymes in fishes. Water sampling analysis from regions around Mumbai and Thane regions has revealed the presence of Nicotine metabolites like norcotinine in water. This provides as evidence that the fishes in these waters are constantly exposed to varying concentration of these compounds, thus undergoing certain physiological changes. As liver is one of the major organs in fishes that aids in detoxifying the system, the present study was aimed at understanding the detrimental effects of Nicotine on the histopathology of liver in *Labeo rohita* a commercially available food fish. Current observations evidently indicated nicotine as a potential hepatotoxic agent in fishes.

Keywords: Endocrine disruptors, Nicotine, Hepatotoxic agent, *Labeo rohita*.

INTRODUCTION

Nicotine toxicity has become increasingly relevant through marketing and promotions of novel nicotine containing products which from past few decades are freely available in most countries. Cigarettes, bidis and other products containing smokeless tobacco are constantly dumped through sewage in several aquatic environments. Owing to the ubiquitous nature and magnitude of cigarette butts discharged into the environment, studies are needed to determine whether cigarette butt waste can exert ecotoxic effects when in aquatic environments (Slaughter et. al. 2011). Standard textbooks, databases, and safety sheets consistently indicate that the lethal dose of nicotine for adults is 60 mg or less (30–60 mg), leading to safety warnings that ingestion of five cigarettes or 10 ml of a dilute nicotine-containing solution could kill an adult. (Hayes 1982). Furthermore, nicotine is marked to have adverse endocrine disrupting effects on the animal biological system. Studies have indicated that nicotine significantly increase the circulating levels of prolactin and LH and decreases serum level of testosterone and FSH (Oyeyipo et al. 2013). Several studies have also indicated endocrine disrupting properties of nicotine in fishes exposed to waters contaminated with waste products like cigarettes, bidis and tobacco. Bidis are products similar to cigarettes that are also combustible and comprise about five times the nicotine content as compared to regular cigarettes. The main objective of the present study was to elucidate the effects of nicotine from bidis on the liver of Indian major carp *Labeo rohita* (Hamilton, 1822) as it is a major food fish widely consumed all over India which is constantly exposed to nicotine and its metabolites that are disposed off as waste products into aquatic habitats into which these fishes reside.

METHODS

Analysis of pollutants in water from research sites: After careful survey, water sampling sites were finalised and water sampling was done. Samples were collected from two different locations from Thane district namely Ulhas river, Wehele and Thane Creek, Thane region respectively. The latitudes and longitudes of each location was recorded to mark the precise location of sampling sites. Locations were finalised based on the presence of *Labeo rohita*, which were abundantly found in these waters. Evidence confirmed the fishing sites near these locations, where this species is caught and sold in local markets. The main objective of water sampling was to analyse the samples to detect the presence of Nicotine or its metabolites.

Sample collection and feed: *Labeo rohita* was procured from Aarey Fish Farm, Goregaon, Mumbai and for experimental purpose, the sub-adult stage of the fishes (yearlings) were finalised for study and observation. The approval for this study was sought from the CPCSEA. Experiments were conducted for 30 days of which 8 days was the Acclimation phase and 21 Days the Experimental phase. The feed consisted of rice bran and groundnut oil cakes crushed into a fine powder in a ratio of 1:1 and was given as 2% of total body weight of the fishes present in each tank. Fishes received this feed for 8 days of the acclimation phase and subsequently, once the fishes were acclimatized to this feed, from day 9, the exposure phase was conducted by administering a dose of Nicotine to the feed. Initial exposure comprised of a mild dosage, thereafter the dose was gradually increased throughout the Exposure phase of 21 days. Fishes from the Controlled Tanks continued to receive the same feed without the dose. The initial dose was confirmed based on the previous LC_{50} values attained for nicotine.

Histological examination: Histological analysis was performed on tissues affected with BPA. The organs were carefully dissected from the fishes after completion of the exposure period or post mortality. Liver lobes were separated from under the ventral region of the heart. The tissue samples were preserved in 10% formalin and dehydrated by treating with ascending grades of alcohol. Liver tissues were cleared with xylene and impregnated with wax. All sections of the liver tissue were cut 5µm thick. They further underwent systematized staining with Hematoxylin and Eosin (H & E) stains. All slides were carefully examined after staining and photographs were recorded with a high resolution camera from the light microscope at power 10X and 45X respectively.

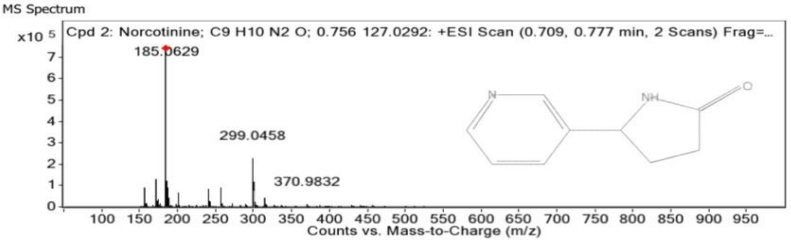
OBSERVATIONS AND RESULTS

The water samples were processed under HR-LCMS (High Resolution Liquid Chromatography Mass Spectrometry) in order to detect the target endocrine disrupting compounds in the sample. This technique combines High Performance Liquid Chromatography (HPLC) a powerful analytical and separation technique along with mass spectroscopy, which is a powerful analysis and detection technique. A Qualitative Compound Report (Fig A) demonstrated various array of compounds found in both samples of which Norcotinine, a nicotine metabolite was detectable in trace amounts indicating *Labeo rohita* is constantly exposed to nicotine contamination. Initial dose was commenced at LC_{50} for Nicotine was attained at 0.78ppm. Normal architecture of the liver of *Labeo rohita* comprised of a continuous group of cells called hepatocytes. They indicated densely granulated cells with centrally located nuclei. Bile duct and vessels were observed amid scattered connective tissue surrounding hepatic cells. The central vein and hepatocytes were separated by mass of blood sinusoids within the liver parenchyma. Portal vein appeared normal in control fishes. Fig I, II and III are the Control liver tissues that indicate the presence of normal hepatocytes and other organelles. However, the hepatocytes of nicotine- affected fishes appeared extremely vacuolated and damaged in the hematoxylin and eosin stained sections. Liver tissues exposed to nicotine as seen in Fig III, IV, V, VI, VII, VIII and IX indicate that

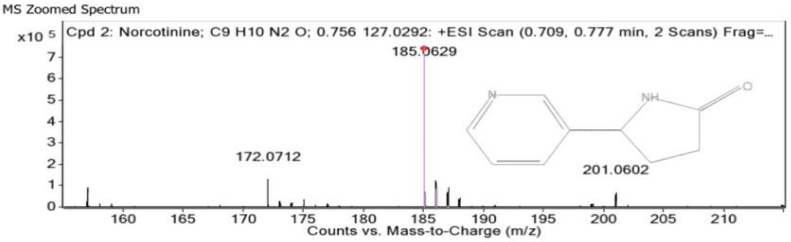
hepatocytes lacked an assorted arrangement due to toxic effects of nicotine leading to acute necrosis in major areas and vacuolation. Disintegrated cluster of necrotic tissues were also observed in some sections along with major damage to vital organelles like bile duct and portal vein.

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 2: Norcotinine; C9 H10 N2 O; 0.756 127.0292	**Norcotinine**	185.0685	0.756	Auto MS/MS	162.0793

MS Spectrum



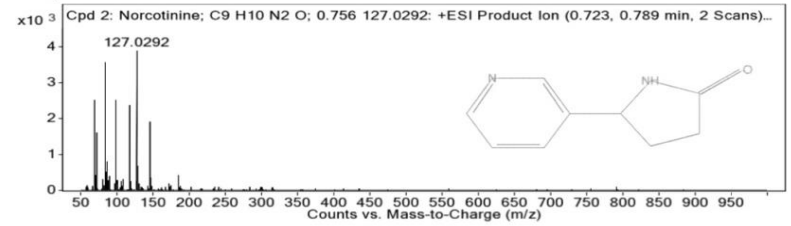
MS Zoomed Spectrum



MS Spectrum Peak List

m/z	*Calc m/z*	Diff(ppm)	z	Abund
172.0538			1	102026.37
172.0712			1	129471.19
185.0629	162.0788	-124196.3	1	766716.5
185.0803			1	404510.35
186.065			1	97220.18
186.0821			1	119799.54
299.0458			1	230217.55
299.0684				227575.44
300.069				115840.6
301.0658			1	105536.5

MSMS Spectrum



MS/MS Spectrum Peak List

m/z	Abund
68.9841	2534.99
83.0254	3552.01
83.0374	3386.06
99.032	2579.78
117.0288	2016.15
117.0434	2457.44
127.0133	3266.1
127.0292	4006.04
145.0267	1890.62
145.0436	1972.35

Compound Structure

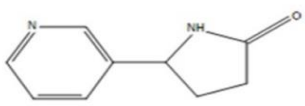


Fig A: Qualitative compound report indicating the detection of norcotinine, a nicotine metabolite in water sample analysis.

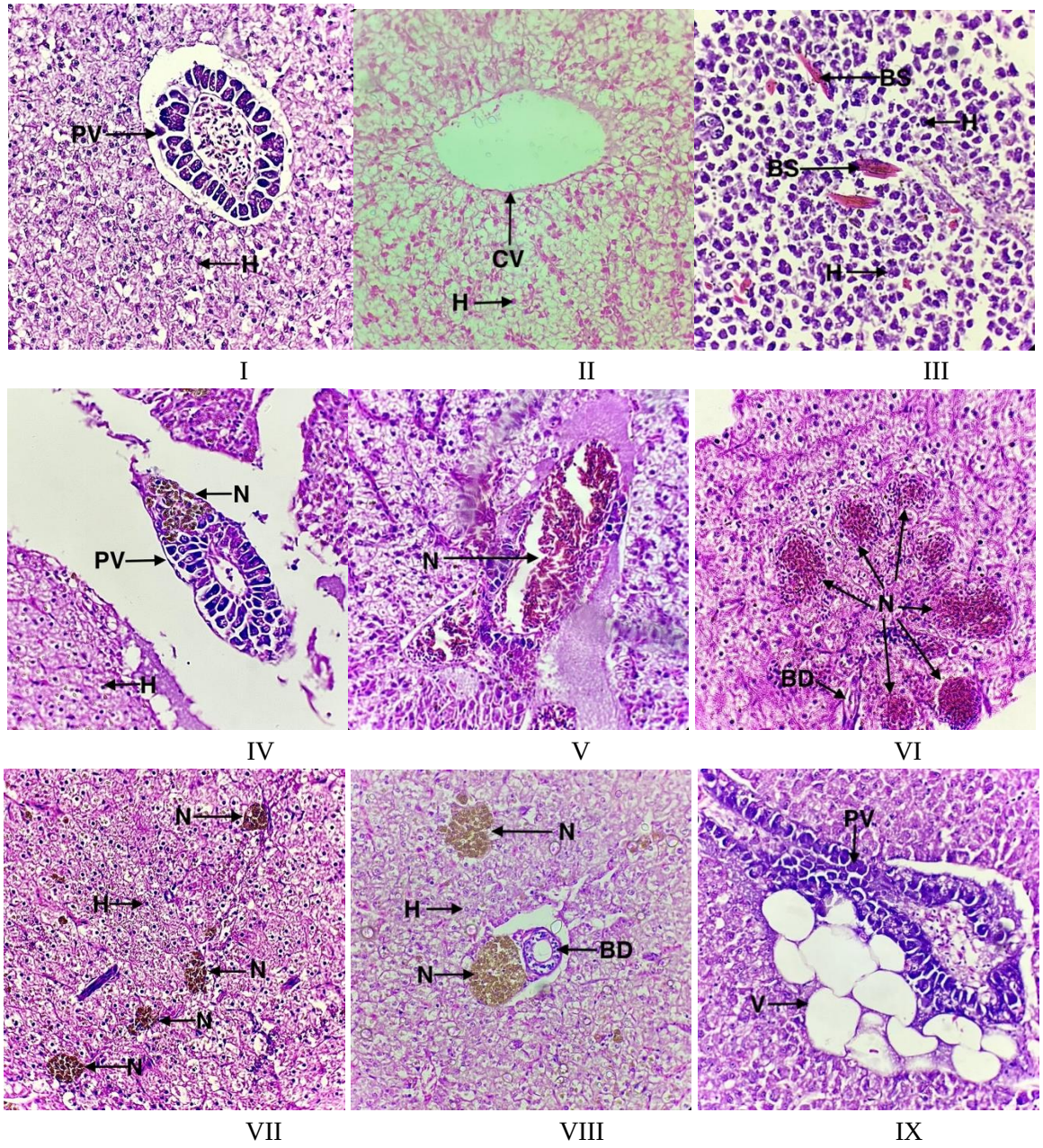


Fig I, II, III: Liver sections of control fish showing normal histological structures.
Fig IV, V, VI, VII, VIII, IX: Liver sections of fishes treated with nicotine;
Pv: Portal Vein, BD: Bile duct, CV: Central vein, BS: Blood sinusoids, H: Hepatocytes, N: Necrosis, V: Vacuolation. (HxE Stain; 45X).

DISCUSSION AND CONCLUSION

Much of the research has been previously carried out on the potent effects of nicotine. According to Omoniyi et. al. 2002, who worked on effect of lethal and sub-lethal concentrations of tobacco leaf dust extract in *Clarias gariepinus*, suggested that the acute toxicity test led to mortalities in the treated fish. Findings of Slaughter et. al. 2011, who worked on toxicity of cigarette butts and their chemical components, to marine and freshwater fish, revealed an increase in the toxic components of a burnt cigarette tip due to its combustion. Studies carried by Kupekar et al. 2015, demonstrated the effect of cigarette smoke on liver in *Carassius auratus auratus* an aquarium

fish. The observations revealed histopathological alterations like hypertrophy of hepatocytes and dilation of sinusoids. Other effects were severe vacuolation and degenerative necrotic condition. Experiments by Konar et. al. 1980, who worked on Toxicity of Nicotine to Aquatic Life indicated that liver damage was a well-known effect of nicotine observed on fish. Similarly, experiments by Ravichandran et. al. 2022, illustrated increased amino acid content in liver of sub-lethal concentration of nicotine-exposed *Labeo rohita* fingerlings along with a decline in liver concentrations of ACP and ALP enzymes.

The present research focused on the deleterious effect of Nicotine on the liver of *Labeo rohita*. Histopathological analysis revealed the effect of nicotine causing certain impairments in the hepatocytes. The initial dosage of 0.012mg/L led to minor aggregation of necrotic tissues. Disruption of endothelial cells, hepatocytes and blood sinusoids along with necrotic cells aggregating around bile ducts and portal veins was observed with a gradual increase in dose at 0.7mg/L. Additionally, multiple clusters of disintegrating cells as a result of the onset of necrosis were also evaluated in some other major parts of the liver sections.

Overall it can be concluded that nicotine plays the role of a potential hepatotoxic agent in fishes. Although previous studies indicate detrimental effects of nicotine on physiology of several fishes, the current work demonstrating effect of nicotine on liver histopathology of *Labeo rohita* is the first work of its kind. As *Labeo rohita* is a major Indian carp and a favored food fish widely consumed all over India, the adverse effects post consumption of affected fishes on higher vertebrates are yet to be elucidated.

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