India is an agrarian country and 16% of its total GDP is contributed by agriculture. She contributes 20% of all world rice production\(^1\). For better growth and production, agricultural crops need a comprehensive cover of crop protection chemicals (CPCs)-organically based traditional or modern synthetic pesticides. Being cost effective and more efficient, synthetic pesticides have competed the traditional methods used to protect crop damages due to insect, pest, diseases & weeds.

India ranks second in Asia for pesticide production and tenth in world as its total consumption amounts to about 500 million tonnes\(^2\). In India, 51% of food supplies are contaminated with pesticide residues and out of these, 20% have pesticide residues above the maximum permissible level\(^3\). The insecticides consumption in India accounts for about 80% of total pesticide consumption\(^4\). Besides, affecting targeted pests, pesticides used in agricultural fields also adversely influence a wide range of non-target aquatic organisms like invertebrates and fish\(^5-6\).

The Carbofuran (CF) (2, 3-dihydro-2, 2-dimethylbenzofuran-7-yl-methylcarbamate) is an insecticide of carbamate group and marketed under the trade names Furadan and Curater. It is a broad-spectrum, ubiquitous, systemic insecticide\(^7\) used for better crop production at large scale\(^8\) particularly paddy and sugarcane\(^9\). On account of its widespread use, carbofuran has been detected in ground, surface and rain waters, and soils\(^10-11\) and is highly toxic to aquatic animals and fishes in particular\(^12\). It inhibits the acetylcholine esterase enzyme activity at synapses in the CNS as well as neuromuscular junctions at sub-acute concentrations\(^13\) and produces hypercholinergic activity of central and peripheral nervous tissues\(^14\). After entering in the aquatic environment through agricultural runoff, CF contaminates the water bodies. CF has been reported to be toxic to several beneficial arthropods\(^15\) and aquatic organisms including different fish species\(^16-17\).

Continuously increasing concentration of CF in the environment is found to be associated with several adverse effects\(^18\). Once this pesticide gets entry into fish body it causes various physical, physiological and biochemical disturbances\(^19\). The alteration of these parameters can be used as sensitive health indicators of the aquatic environment\(^20\). Fish are also excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples since they are frequently exposed to a variety of toxicants and they have the ability to metabolize, concentrate, and store waterborne pollutants quickly\(^21\).

Toxic responses in fishes against xenobiotic stressors can be efficiently recorded and monitored in terms of clinical
hematology and biochemistry besides genotoxic studies. Blood is extremely sensitive to internal and external environment perturbations as it is continuously involved in the transport of various substances including pollutants\textsuperscript{22-23}. Haematological parameters are one of the most sensitive indicators of physiological disturbances\textsuperscript{24} thus, they can be used as indicator of fish health status to detect physiological changes following different stress condition like exposure to pollutant, diseases, hypoxia\textsuperscript{25} etc.

Similar to haematological parameters, the biochemical parameters such as enzyme levels, altered during toxic exposure, also provide an idea for toxicity assessment \textsuperscript{26}. Researches indicate that liver enzymes can be used as biomarkers of cellular damage in blood plasma, protein degradation and liver damage itself\textsuperscript{27-29} and therefore the activities of liver marker enzymes viz: Aspartate Transaminase (AST), Alanine Transaminase (ALT) and Alkaline phosphatase (ALP) can be used efficiently for assessing the xenobiotic stress\textsuperscript{29} as well as protective role of phytochemicals\textsuperscript{27}.

In addition to biochemical and haematological toxicity, genotoxic risks generated by pesticides in animals are also significant, so that comprehensive toxicological profile of the animal under xenobiotic stress can be worked out. Genotoxicity of toxicants are governed by a variety of factors viz., their stability or biological accumulation in the environment, their in vivo metabolism, and their action on biomolecules viz., DNA, RNA and proteins\textsuperscript{30}. Besides the high stability of CF, its toxicity also results in the production of free radical mediated neural cells damage\textsuperscript{31}. Furthermore, the free radicals have been widely reported to generate genotoxicity due to their ability to bind biomolecules\textsuperscript{32}. Basic molecular technique, Random Amplified Polymorphic DNA (RAPD) can be effectively utilized here to detect xenotoxic damage. RAPD depends on amplification of random segments of genomic DNA using the polymerase chain reactions\textsuperscript{33}. Since it is a simple technique and does not need prior knowledge of target sequence, this tool has ample application in primary assessment of DNA damage induced by genotoxic chemicals\textsuperscript{34-35}. Further, the main advantages of the RAPD technique is its rapidity and high sensitivity to detect DNA damage and mutations\textsuperscript{36} therefore, this can be effectively use to study genotoxicity in fish induced by pesticides.

**MATERIAL AND METHODS**

**Experimental procedure: Fish acclimatization:** Healthy and live fresh water teleostian fish, *Channa punctatus* (15 ± 1.0 cm and 30 ± 2.0 g) were hand netted from local lentic habitats in the vicinity of Lucknow. They were given prophylactic treatment in formalin (0.4%) for 5 min followed by KMnO\textsubscript{4} (1 mg l\textsuperscript{-1}) for 30 minutes to keep away dermal infections, if any. Fish were acclimatized under laboratory conditions (Temperature, 14 to 22°C, dissolved oxygen, 6.62 to 6.76 mg/l, alkalinity, 62 to 68 mg/l) for 10 days in large glass aquaria (100x40x40 cm\textsuperscript{3}) before experimental exposure. During acclimatization period, standard methods for fish maintenance procedure were maintained by following the standard procedure (APHA et al.,). During experiment they were fed with minced goat liver and artificial fish food.

Technical-grade Carbofuran (CF) 3% G with product name Furadan 3 G (manufactured by FMC India private Ltd., Hawrah W. B. India) was purchased from the local dealer. The value of 96 hr-LC50 of CF was determined as 0.9mg l\textsuperscript{-1} by Trimmed Spearman-Karber Method\textsuperscript{38}. Based on 96 hr-LC50 value, the sub-lethal fraction of CF i.e., 96 hr LC50/10 was estimated 0.09 mg l\textsuperscript{-1} and used for in vivo experiments. The dried roots of *Rauvolfia Serpentina* (Sarpagandha) were purchased from market of Lucknow. 40g of powdered root was extracted in soxhlet apparatus with ethanol (1 L; 95%) for about 12 hr at 25°C. After 12 hr, filtrate was concentrated till semi solid state was achieved by rotary vacuum evaporator. This semi solid ethanolic root extract was stored in refrigerator (4°C) and used for the experiments.

**Experimental design:** Ten days acclimatized fish were divided into 3 groups having 10 fish in each group. Briefly, group 1 was control, group 2 was treated with 96 h-LC50/10 fraction of CF alone and group 3 was treated with 96 h-LC50/10 fraction of CF along with 10 ppm concentration of Sarpagandha (SPG). After the end of stipulated exposure periods viz, 24, 48, 72, and 96 hr. Fish were randomly selected for collection of blood, from all groups. Triplicates were used for each group to verify reproducibility of experimental results.

**Haematological assay:** For the estimation of hematological parameters blood was collected from caudal vein (OECD 2005) and stored in 5ml vials coated internally with anticoagulant 0.5% EDTA. Total Leucocyte Count (TLC), Hemoglobin
percentage (Hb%), Packed Cell Volume (PCV), Erythrocyte Sedimentation Rate (ESR), and Clotting Time (CT), were assessed by employing standard methods.

Biochemical assay: The biochemical parameters in terms of liver marker enzymes such as AST, ALT and ALP were determined by Ecoline diagnostic kits (Merck No.1176740011730; 1176630011730; and 11767700011730, respectively).

Random Amplified Polymorphism (RAPD) Analysis: For RAPD analysis, genomic DNA was isolated from fresh blood by using the phenol-chloroform extraction protocol. The concentration and purity of DNA was determined by measuring the absorbance of diluted DNA solution at 260nm and 280nm by spectrophotometer (UV/visible-1800 Shimadzu). Random primers with 10 bases were used for DNA amplification by thermocycler (eppendorf flexlid mastercycler). PCR amplification was carried out in 25µl reaction volume containing 50ng genomic DNA, 100µM dNTPs, 40 nM primer (Operon), 2.5 units of Taq DNA polymerase and 5µl promega 10X Taq DNA polymerase buffer. PCR cycling conditions were initial denaturation of 5 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C and finally, one cycle at 72°C for 5 minutes. Amplified products resolved on 1.5% agarose gel, were observed under UV illumination and images were captured with a Gel Documentation System (Vilber Lourmat).

RESULTS AND DISCUSSION

Heamatological parameters viz. clotting time (CT), TLC, Hb%, ESR and PCV were evaluated for different experimental groups and were presented in Figs.-1.A to 1.E. The present study demonstrated a time dependent reduction in the clotting time of blood of group 2 fish which were exposed to 1/10th fraction 96 hr LC50, in comparison to control group fish which were not exposed to any toxicant. This reduction in CT of blood was found to be highest (i.e. from 56±2.70 sec. in control group fish to 52±1.2 sec. in group 2 fish) at 96 hr of exposure period. However, this reduction of blood CT in fish of group 3 has been found to be less evident and it is 55±1.11 sec., quite close to CT value recorded in blood of fish of control group. The group 3 fish were exposed with 96 hr-LC50/10 fraction of CF along with 10 ppm concentration of SPG. The total leucocyte count (TLC) was gradually enhanced in time dependent manner on exposure of cabofuran in group 2 in comparison to control group fish from 68±2.22 per cubic mm to 86±3.2 per cubic mm, while after Sarpagandha treatment along with CF in group 3, TLC of blood of fish (70±2.32 per cubic mm) was less affected and was recorded to control value. Hb% was recorded decreasing throughout the exposure periods in Carbofuran treated fish from group 2 in comparison to Hb % of control group fish i.e. 10.6±0.22 and the highest decrease (7±0.21) was recorded after 96hr exposure period. This decrease was not pronounced i.e. 9±0.54 and was recorded close to control value in blood of fish of group 3 in which fish were treated with CF and SGF simultaneously. ESR values were recorded increasing throughout the exposure periods in Carbofuran treated fish from 8.0±0.27mm/hr to 9.1±0.23mm/hr but with simultaneous treatment with Sarpagandharoot extract. ESR values were estimated 8.55±0.34mm/hr i.e. close to normal value (8.0±0.27). PCV values were recorded decreasing to 28.4±0.22 in blood of fish of Carbofuran treated group 2 from 34.3±0.21 in blood of fish of control group 1. On the other hand, simultaneously Sarpagandha root extract and Carbofuron treated group 3, PCV values were observed close to normal value 30.6±0.42. Thus, some haematological parameters viz., Hb%, PCV, and CT were reduced while other parameters like TLC and ESR were increased in CF treated fish. The alteration in haematological indices may be due to an appreciable decline in the haematopoiesis endorsed by CF toxicity.

Hematological parameters are directly related to the response of the animal to the pollution status of habitats where fishes live Gabriel et al., has stated that the fall in haemoglobin concentration is due to decreased erythrocyte count on exposure of pesticides. Similar reduction in Hb% and TLC in carbofuran treated group was also recorded in the present study that may be due to the toxic effect of carbamate pesticides. Similar perturbations in haematological parameters

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<th>S.No.</th>
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<tr>
<td>1.</td>
<td>OPA-01</td>
<td>5’ CAG GCC CTT C 3’</td>
</tr>
<tr>
<td>2.</td>
<td>OPA-02</td>
<td>5’ TGC CGA GCT G 3’</td>
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<td>3.</td>
<td>OPA-03</td>
<td>5’ AGT CAG CCA C 3’</td>
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<td>4.</td>
<td>OPA-13</td>
<td>5’ CAG CAC CCA C 3’</td>
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FIGURE-1. A-E: Representation of different haematological parameters viz., CT, TLC, Hb%, ESR, and PCV of blood of *C. punctatus* in different experimental groups after different exposure periods viz., 24hr., 48hr., 72hr. and 96hr.

on exposure of quinalphos pesticide in silver barb fish have also been documented\(^6\). Changes in these haematological constraints are directly related to toxicity induced by toxic chemicals\(^9\), pollutants and other environmental factors\(^2\). The toxic effects of carbofuran were not pronounced in group 3 where fish were treated by carbofuran along with Sarpagandha root extract and this can be attributed by its medicinal effects on fish health. Likewise, Azmi and Qureshi\(^22\) have documented the ameliorative potential of Sarpagandha root extract on blood parameters of Alloxan treated mice.

Several biochemical and physiological alterations occur when a toxicant enters into an organism\(^4\). Carbofuran mainly works by inhibiting the activity of the enzyme acetylcholinesterase \(^4\), which is an important enzyme in nerve impulse transmission. Trotter\(^16\) have reported that carbofuran is extremely toxic to fish and its 96 hr LC50 is less than 1 mg/L. Biochemical values in terms of activities of liver enzymes viz., AST, ALT and ALP were found increased after exposure to carbofuran in group 2 in a time dependent manner. However, insignificant increase was recorded in group 3 fish, exposed to carbofuran along with Sarpagandha (fig. - 2.A to 2.C). The observed increase in the activity of AST was recorded from 17.16 ± 1.068 IU/L in blood of control group fish to 37.74 ± 0.697 IU/L in blood of carbofuran treated group 2 fish. However, this increase was insignificant, near to control value (26.61 ± 1.069 IU/L) in fish treated with Sarpagandha along with carbofuran in group 3.

Likewise, inductive pattern of ALT activities from 22.48 ± 1.004 IU/L in control group fish to 39.24 ± 0.708 IU/L in carbofuran exposed group 2 were recorded. This increase was more evident in the group 2 where no Sarpagandha treatment was given as compared to fish of group 3 treated with Sarpagandha along with Carbofuran (32.71 ± 1.004 IU/L). Furthermore, ALP
FIGURE-2. A-C: Representation of different Liver marker enzyme viz., AST, ALT and ALP of blood of *C. punctatus* in different experimental groups after different exposure periods viz., 24hr., 48hr., 72hr. and 96hr.

The hepatotoxic chemical such as pesticide disturbs the
normal hepatic physiology but they can be maintained by hepatoprotective agents. Phytochemicals are partly responsible for the health benefits through a variety of mechanisms. Present study justifies that the ethanolic extract of Sarpagandha root extract possesses ample hepatoprotective effect. In fact, the antioxidant phytochemical compounds present in Sarpagandha root extract are responsible for its hepatoprotective effect. Similar biochemical findings are also observed by Khan et al.

The differential genomic patterns of the amplified DNA generated by using random primers was evaluated for the fish Channa punctatus in control as well as treated groups. Primers were used to amplify the random sequences and clearly detectable bands were recorded on agarose gel (Fig.-3). These random primers amplified and results obtained were varied from 130 to 1250 base pairs in the form of bands. On average 8.3±0.9 bands per primer were obtained. Maximum score-able bands were similar and monomorphic for control and treated groups. Moreover, most of these bands were monomorphic for fish of control group and group 3 in which fish were co-exposed with Sarpagandha and Carbofuran, whereas bands were not similarly monomorphic in case of carbofuran treated group 2 fish. The DNA of the samples of fishes this group treated with carbofuran reveals the appearance of average 30% new bands (polymorphic) at 96 hr of exposure period which did not appear in the samples of control group. These new bands could be considered as genetic diagnostic markers which attributed by Carbofuran toxicity. In addition, the results clearly reflect that primers used herein show appearance of different polymorphic bands in a time dependent manner in different treated groups. The appearance of bands were obtained in group 3 is almost close to the control group which indicates the protective effects of Sarpagandha root extract in terms of genetic diagnostic marker against the CF induced impairments in fish. Azami and Quereshi reported the presence of alkaloids, carbohydrates, flavonoids, glycosides, cardiac glycosides, phlobatannins, resins, saponins, steroids, tannins, and triterpenoids in root extract of Rauvolfia Serpentina. These compounds may be attributed to impart ameliorative role in counteracting toxicity generated by CF in C. punctatus. Khan and Sinha have also reported vitamin C induced dose-dependent amelioration of genotoxicity induced by the pesticides in mice. Similar findings are also reported for DNA damage in terms of comet assay in rat against carbofuran toxicity and their recovery by secondary metabolites present in Ipomoea aquatica.

The hematological, biochemical and genotoxic investigations are indicative of the fact that adverse effects of Carbofuran pesticide on fish health can be effectively encountered by remedial action of Sarpagandha root extract. Thus, this study provides toxicological responses of Carbofuran and remedial role of Sarpagandha root extract on three major physiological departments of fish, viz., haematological, biochemical and molecular. Since, there is much concern about the toxicity of pesticides to the native fauna, it is expected that the optimization and cultivation of plant species that produce active ingredients as secondary metabolites (Alkaloids, flavonoids and polyphenols etc.) will be beneficial for the aquaculture industry and consequently be more profitable and safe for fish production. We can conclude that Carbofuran has harmful effects on fish metabolism and its genetic integration. Several studies indicate that some plant extracts including Rauvolfia serpentina showed an ameliorative activity due to its richness in secondary metabolites. Thus, generated data and inferences in present study will be helpful not only for pollution biologists but also for fishery farmers to protect their fishery resources against carbofurantoxicity by using naturally occurring plant products. Outcome of the study may settle a milestone for fishery biologists and policy makers in their efforts to conserve fish biodiversity which is the need to overcome the challenges of population explosion and concomitant shrinking of traditional agricultural resources, viz., land farming.

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