Introduction

Furadan 3G (2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl methylcarbamate) is used as a broad spectrum systemic carbamate pesticide for agriculture. Although, it is already banned in the United States and some European countries (Ruiz-Suárez *et al*., 2015), it is still used as a potent crop protection chemical in most of the Indian states (Mishra and Sharma, 2011). Furadan 3G is one of the preferred pesticides used to protect crops by killing insects, nematodes and parasites (Wandan *et al*., 2011). As a result of its widespread use, furadan 3G has been detected in ground, surface and rain water, air and food (Nagaraja and Bhaskara, 2006; Waite *et al*., 1992). Pesticides used in the agricultural fields are mobilized and get deposited in the aquatic ecosystem through surface run-offs (Gnanavelrajah and Kandasamy, 2012). When furadan 3G like pesticides is used in paddy-cum fish culture, it causes health risk to fish due to their direct exposure. By consuming these unhealthy fish, humans are also at risk of variety of diseases (Zeljezic *et al*., 2008).

Furadan 3G has also been documented as a teratogenic, embryotoxic (Gupta, 1994) and genotoxic compound (Chauhan *et al*., 2000; Naravaneni and Jamil, 2005; Zeljezic *et al*., 2007). Exposure of furadan 3G causes various genotoxic, biochemical and endocrine anomalies in organisms like micronuclei formation in mice (Chauhan *et al*., 2000). Furadan 3G in particular, impairs nerve transmission by inhibiting enzyme acetylcholine esterase (AChE) (Usmani *et al*., 2004; Zeljezic *et al*., 2008). Acetylcholinesterase inhibition generates oxidative stress due to increased production of free radicals (Milatovic *et al*., 2006). In addition to affecting enzymes, furadan 3G generated free radicals may alter the property of DNA, RNA and proteins (Wu and Cederbaum, 2003). Hence, application of pesticides as crop protection chemical poses inevitable risk for human health; therefore a search for suitable measures that can counter their ill effects is desirable.

Eco-friendly approach by using natural remedy to protect crops is the need of the hour. Several plant secondary metabolites are known to possess an effective antioxidative potential and have been reported to counter toxicity generated by xenobiotics (Dai and Mumper, 2010; Sowndhararajan and Kang, 2013). *Melissa officinalis* is a perennial herb and has been reported for its excellent anti-oxidative properties (Dastmalchi *et al*., 2008; Martins *et al*., 2012). The characterization of *Melissa officinalis* extract showed several polyphenolic compounds *viz*., rosmarinic acid, protocatechuic acid, caffeic acid and essential oil like citral; and various other phytochemicals *viz*; sesquiterpenes, monoterpenoid, potential antioxidants, tannins and flavonoids (Carnat *et al*., 1998; Guginski *et al*., 2009). Melissa officinalis has been adequately screened for its anti-oxidative properties using various anti-oxidative assays *viz*., DPPH, DMPD etc. (Atanassova *et al*., 2011; Guginski *et al*., 2009). Considering the above facts, in the present investigation the protective role of ethanolic extract of *Melissa officinalis* was screened in terms of cytotoxic end points-micronuclei induction and the activities of marker enzymes of liver, the prime site of biotransformation of xenobiotics in fish.

Materials and Methods

Animals and treatment : Healthy and live fresh water teleostian fish, *Channa punctatus* (14.5 ± 1.0 cm and 30 ± 2.0 g) were procured from local lentic habitats of Lucknow, India. They were given prophylactic treatment of formalin (0.4%) for 10 min, followed by $KMnO_4$ (1 mg l^{-1}) for 1 hr to keep away the dermal infections. Fish were acclimatized for 10 days in large glass aquaria (100x40x40cm^3) prior to experimentation. During acclimatization period, fish were maintained in standard laboratory conditions (Temperature: 14-22°C, dissolved oxygen: 6.62 - 6.76 mg l^{-1} and alkalinity: 62- 68 mg l^{-1}). Fish were fed with minced goat liver and artificial fish food, Tokyo.

Collection and preparation of plant extract : The whole plant of *Melissa officinalis* was collected from local agricultural fields of Lucknow, India, which was identified by the experts from the Department of Botany, University of Lucknow, Lucknow. The collected plant material of *Melissa officinalis* was washed thoroughly in tap water and dried at room temperature (31°C±2°C) in shed for a minimum of 15 days. After drying, plant material was powdered with the help of an electric grinder. The powder of *Melissa officinalis* was soaked in 50% aqueous-ethanol for one week and then the filtered extract was concentrated to semi solid state by vacuum evaporator. The concentrated semi solid ethanolic extract of *Melissa officinalis* so obtained was stored in refrigerator at 4°C for further experimental work.

Determination of median lethal concentration of furadan 3G : For determination of 96 hr-LC_{50} of furadan 3G in fish, different set of fish were exposed to different logarithmic concentrations of furadan 3G prepared in non-toxic acetone (Pickering *et al*., 1962). Proper oxygenation in aquaria was maintained with the help of stone diffusers. The mortality of fish was recorded at time intervals of 24 hr, 48 hr, 72 hr, and 96 hr, and dead fish were removed immediately. Experiments were repeated thrice to verify their reproducibility. The value of 96 hr-LC_{50} of furadan 3G for fish *Channa punctatus* was determined by Trimmed Spearman-Karber Method (Hamilton *et al*., 1978)

Experimental design : Ten day acclimatized fish were divided into three groups taking ten fish in each group. Among these, group 1 served as control; group 2 was exposed to 1/10th concentration of 96 hr-LC_{50} of furadan 3G (0.09 mg l^{-1}) while group 3 was exposed to 1/10th concentration of 96 hr-LC_{50} of furadan 3G (0.09 mg l^{-1}) along with 10 ppm of *Melissa officinalis*. At the end of the specified exposure periods for 24, 48, 72, and 96 hrs, blood samples were taken by caudal vessel puncture using disposable syringes and kept in vials without using any anticoagulant and smears were prepared at the same time to observe micronuclei (MN) induction before coagulation. Remaining blood was left to stand at room temperature to clot for 30 - 40 min and then

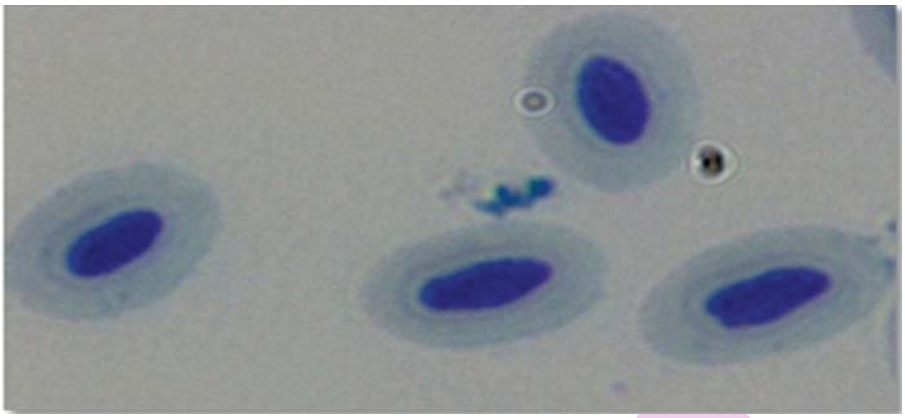

Fig.1 : Normal erythrocytes of *Channa punctatus*

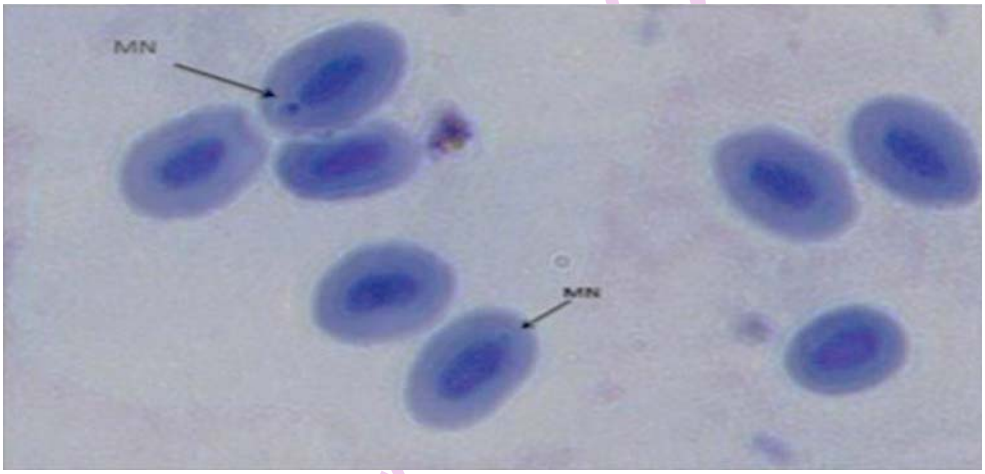

Fig.2 : Micronuclei in erythrocytes of *Channa punctatus*

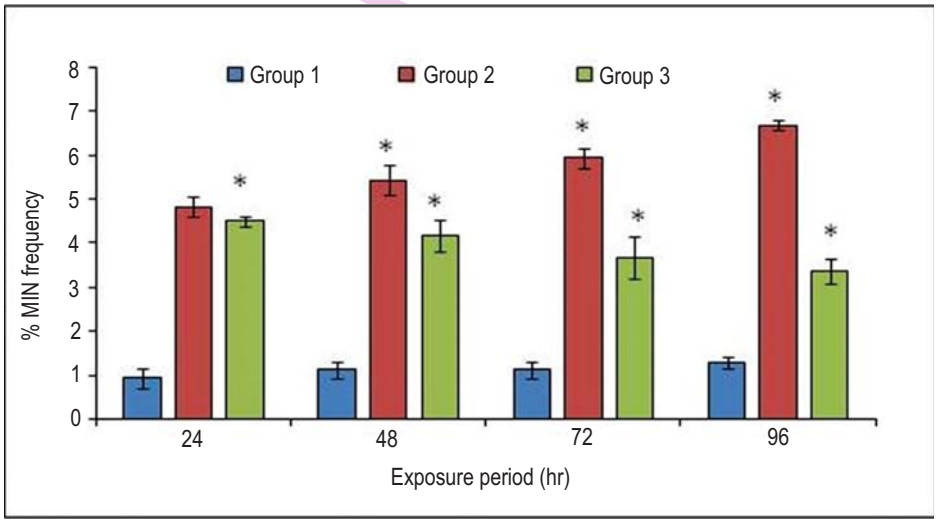

Fig. 3 : Graphical representation of alterations in micronuclei frequencies in erythrocytes of *Channa punctatus* in different experimental groups after 24, 48, 72 and 96 hrs. exposure periods. The Group 1 represents Control group without any exposure, while Group 2 and 3 represent treatment groups exposed with 96hr-LC_{50}/10 furadan 3G and 96hr-LC_{50}/10 furadan 3G+10 ppm *Melissa officinalis*, respectively. * Values are statistically significant at $p<0.05$ level with respect to the control

centrifuged at 3000 rpm for 10 min to obtain serum for enzyme activity analysis from each group of fish.

Micronuclei assay : Peripheral blood samples were smeared on cleaned microscopic slides and fixed with absolute methanol for 5 min. After the fixation step, slides were stained with May-Grunwald's solution 1 and solution 2 for 3 and 5 min, respectively followed by 5% Giemsa staining of slides for 30 min. staining protocol was used by following the standard protocol. Overnight dried slides were further mounted with DPX and observed under oil immersion microscope (Nikon Corporation K 12432) using 40/100X objective lenses. A minimum of 1000 erythrocytes for each specimen were examined. The scoring of micronuclei were done by following the criteria of Fenech *et al*. (2011).

Estimation of liver marker enzymes : Activity of liver marker enzymes, aspartate aminotransferase (AST) was recorded with the help of Ecoline kit (AST: 11767400011730) by following IFCC method (Bergmeyer *et al*., 1976), alanine aminotransferase (ALT) with the help of Ecoline kit (ALT: 11766300011730) by following IFCC method (Bergmeyer, 1980), while alkaline phosphatase (ALP) was estimated by using Ecoline kit: (ALP:11767700011730) following the method of Belfield and Goldberg (1971).

Statistical analysis : Statistical analyses were performed with the help of SPSS (ver. 20.0) software. For the calculation of significance, one-way ANOVA with post hock analysis was carried out. The acceptance level of significance was taken as $p<0.05$.

Results and Discussion

96 hr-LC_{50} of furadan 3G for *Channa punctatus* : The value of LC_{50} (96 hr) of furadan 3G was determined as 0.9 mg l^{-1} for fish species *Channa punctatus*. For conducting further experiments, $1/10^{th}$ concentration of 96 hr-LC_{50} of furadan 3G *i.e*., 0.09 mg l^{-1} was selected for *in vivo* studies.

Effect of furadan 3G on micronuclei induction : Erythrocytes of healthy *Channa punctatus* were observed as elliptical shape with centrally located nucleus (Fig.1). Along with the centrally located large nucleus, some fractions of similar round structure were also observed by Giemsa staining in erythrocytes, usually considered as micronuclei, which were found in the range of $1/10^{th}$ to $1/3^{rd}$ fractions of actual nucleus size (Fig.2) (Fenech *et al*., 2011). The frequencies of micronuclei induction in erythrocytes of different groups of *Channa punctatus* exposed to furadan 3G is summarized in Fig. 3. Fish exposed to1/10 96 hr-LC_{50} of furadan 3G (Group 2) showed a significant ($p<0.05$; Fig. 2.) increase in the frequency of micronuclei induction in comparison to control groups for different exposure periods *viz*., 24, 48, 72, and 96 hrs. However, the frequencies of micronuclei in group 3 (exposed to $1/10^{th}$ concentration of furadan 3G + 10 ppm of *Melissa officinalis*) were observed significantly ($p<0.05$) reduced in comparison to group 2.

Effect of furadan 3G on activities of liver enzymes : The enzymatic activities of AST, ALT and ALP were progressively increased significantly ($p<0.05$) with the exposure periods in a time dependent manner in group 2, which were treated with furadan 3G. However, in group 3, fish were given treatment of furadan 3G along with *Melissa officinalis* extract and the activities of AST, ALT and ALP were found to be significantly reduced in comparison to group 2 ($p<0.05$; Fig. 4). The reduction of AST, ALT and ALP activities in group 3 were recorded in a exposure dependent manner and maximum reduction was noted after 96 hr of exposure period in comparison to group 2.

Fish erythrocytes contain clearly distinguished, centrally situated nucleus with ample amount of cytoplasm, therefore, any anomalous change in nucleus can be easily detected. In the present study, *Channa punctatus* of group 2 exposed to furadan 3G showed significant elevated levels of micronuclei induction over control (Group 1) in an exposure dependent manner ($p<0.05$). The present results are in agreement with the findings of Gadhave *et al*. (2014) who found an increase in micronuclei induction in erythrocytes of *Labeo rohita* after λ-cyhalothrin exposure. Similar results were also reported by Vera-Candioti *et al*. (2013) in fish after exposure of another carbamate based pesticide, pirimicarb. Increased MN induction was also evaluated by Bhatnagar *et al*. (2016) after acute toxicity of chlorpyrifos exposure in freshwater fish, *Cirrhinus mrigala* and Pandey *et al*. (2014) after profenofos exposure in *Channa punctatus*.

Micronuclei are formed due to several reasons, such as improper chromosomes segregation during meiosis or damaged DNA fragments (Fenech *et al*., 2011). Luzhna *et al*. (2013) demonstrated that spindle failure induced abnormalities during meiosis is the key reason to induce micronuclei formation rather than chromosome breakage. The present study also demonstrates that *Melissa officinalis* treatment along with furadan 3G in group 3 fish showed a significant decline in the frequencies of micronuclei induction in comparison to group 2 ($p<0.05$). The micronuclei induction frequency after 24 hr of exposure in group 3 did not show any significant differences in comparison to group 2, however, in the later exposure periods of 48, 72 and 96 hrs, frequencies of micronuclei induction in group 3 decreased significantly ($p<0.05$) in comparison to group 2, but insignificant as compared to group 1. This exposure dependent reduction in the frequency of micronuclei by applying *Melissa officinalis* has suggested its ameliorative potential against furadan 3G induced genotoxicity. Moreover, in addition to genotoxic insults furadan 3G has been also reported to alter liver biochemistry. The results of the present study showed a significant increase in the activities of liver marker enzymes (AST, ALT and ALP) under the influence of pesticide, furadan 3G. Similar findings of alterations in liver enzymes in blood of *C. gariepinus* exposed to carbofuran doses were observed by Harabawy and Ibrahim (2014).

Pesticides generally affect biologically active molecules *viz*., proteins, carbohydrates and lipids (Singh and Sharma, 1998). In the present investigation, alteration in enzyme level of liver tissues in group 2 animals indicates that a higher activity of liver enzymes is required to detoxify the toxicants introduced in the body under furadan 3G exposure. Enhancement in enzyme level of liver

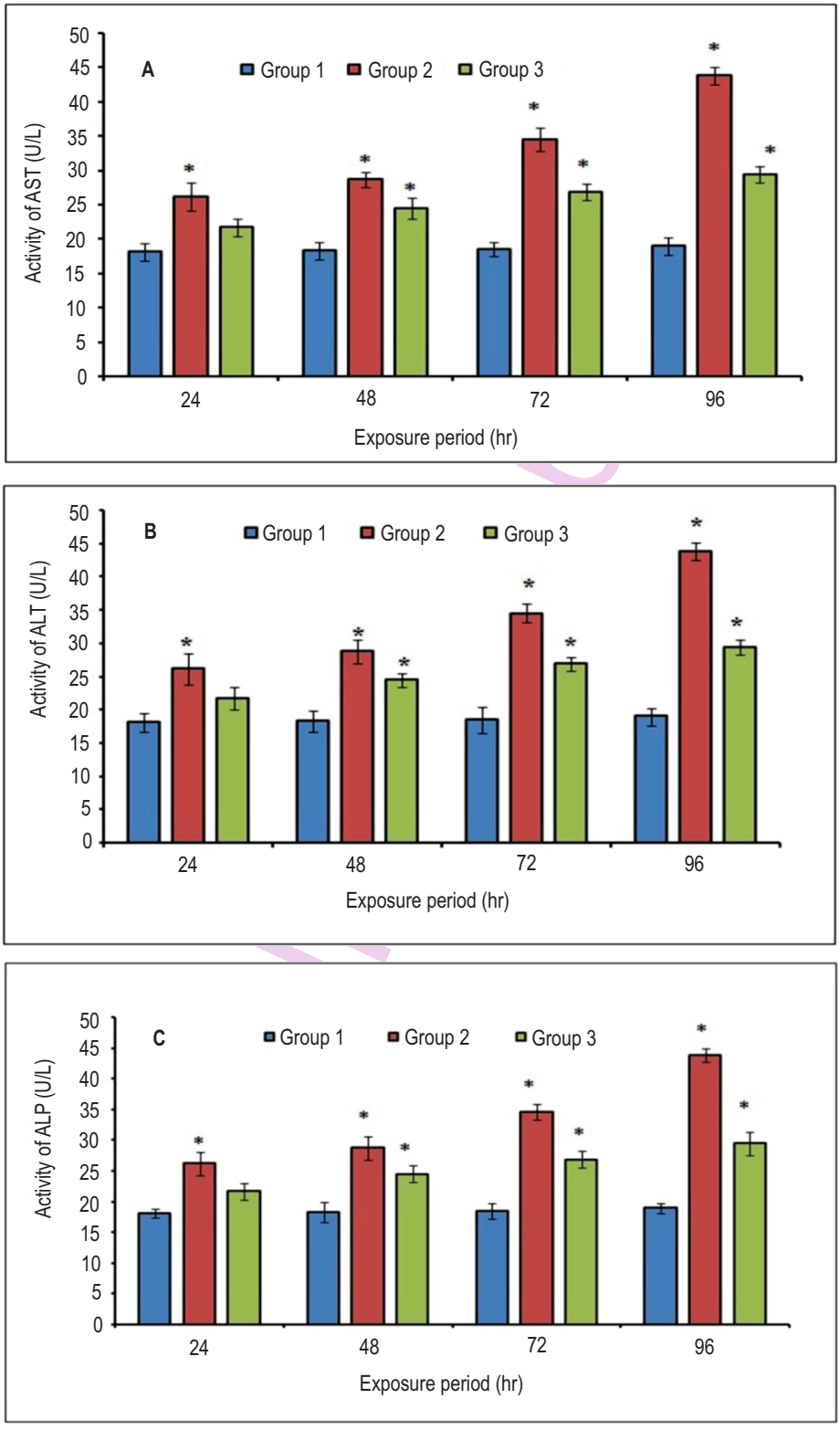

Fig. 4 : (A) Graphical representation of alterations in AST enzyme activities in blood of *Channa punctatus* in different experimental groups; (B) Alterations in ALT enzyme activities in blood of *Channa punctatus* in different experimental groups; (C) Alterations in ALP enzyme activities in blood of *Channa punctatus* in different experimental groups. The Group 1 represent Control group without any exposure, while Group 2 and 3 represents treatment groups exposed with 96hr-LC_{50}/10 furadan 3G and 96hr-LC_{50}/10 furadan 3G +10 ppm *Melissa officinalis*, respectively. * Values are statistically significant at $p<0.05$ level with respect to the control

is part of an important mechanism for transformation and removal of toxic chemicals from the body. Thus, by introducing *Melissa officinalis* extract along with sub-lethal fraction of furadan 3G (group 3), liver enzymes activities reached towards the normal value, which may be due to amelioration of furadan 3G toxicity. *Melissa officinalis* extract is able to suppress the toxic action of furadan 3G, as measured in terms of recovery of micronuclei frequency and level of liver enzymes in group 3 towards control. This protective response of *Melissa officinalis* could be due to its antioxidant property which helps in maintenance of cellular and nuclear health.

Plant extract contains a number of potent antioxidants including flavonoids, phenolic acids and tocopherols and have shown their ameliorative potential against toxic effects of pesticides and other toxicants (Dastmalchi *et al*., 2008). Ameliorative role of *Melissa officinalis* against manganese toxicity has also been demonstrated by Martins *et al*. (2012) in mice. Similar protective role of *Mentha piperita* against benzo[a]pyrene-induced lung carcinogenicity and mutagenicity in Swiss albino mice has been established by Samarth *et al*. (2006). The findings of the present study also demonstrate the protective response of *Melissa officinalis* in fish *Channa punctatus* treated with sub-lethal concentration of furadan 3G. evident by a significant reduction in the activities of liver marker enzymes AST ($p<0.05$), ALT ($p<0.05$) and ALP ($p<0.05$) and induction of micronuclei ($p<0.05$), a cytotoxic end marker. Thus, the plant extract in reference can be effectively utilized for fish health management in aquaculture practices, particularly in paddy-cum fish culture where carbofuran application is more prevalent.

Acknowledgments

One of us, Vidyanand Tiwari, gratefully acknowledge the UGC, New Delhi, India for providing financial support in form of Major Research Project (Award no. F-37-426/2009 (SR). Authors are thankful to the Head, Department of Zoology, University of Lucknow, India for providing necessary facilities.

References

Atanassova, M., S. Georgieva and K. Ivancheva: Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. *Ivancheva J. Univ. Chem. Technol. Metall.*, **46**, 81-88 (2011).

Belfield, A. and D.M. Goldberg: Colourimetric determination of alkaline phosphatase activity. *Enzyme*, **12**, 561-568 (1971)

Bergmeyer, H.U., G.N. Jr. Bowes, M. Jr. Horder and D.W. Moss: Provisional recommendations on IFCC methods for the measurement of catalytic concentrations of enzymes. Part 2. IFCC method for aspartate aminotransferase. *Clin. Chem. Acta*, **70**, F19-F42. (1976)

Bergmeyer, H.U.: IFCC methods for the measurement of catalytic concentrations of enzymes. Part 3. IFCC method for alanine aminotransferase (l-alanine : 2-oxoglutarate aminotransferase) *Clin. Chem. Acta*., **105**, 147-154. (1980)

Bhatnagar, A., A.S.Yadav and N.Cheema : Genotoxic effects of chlorpyrifos in freshwater fish *Cirrhinus mrigala* using micronucleus assay. *Advances in Biology*, **2016**, (2016).

Carnat, A.P., A. Carnat, D. Fraisse and J.L. Lamaison: The aromatic and polyphenolic composition of lemon balm (*Melissa officinalis* L. subsp. officinalis) tea. *Pharm. Acta Helv*.,**72**, 301-305 (1998).

Chauhan, L.K., N. Pant, S. Gupta and S. Srivastava: Induction of chromosome aberrations, micronucleus formation and sperm abnormalities in mouse following carbofuran exposure. *Mutation Research*, **465**, 123-129 (2000).

Dai, J. and R.J. Mumper. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*, **15**, 7313-52. (2010)

Dastmalchi, K., H.J. Damien Dorman, P.P. Oinonen, Y. Darwis, I. Laakso and R. Hiltunen: Chemical composition and *in vitro* antioxidative activity of a lemon balm (*Melissa officinalis* L.) extract. *LWT - Food Sci. Technol*., **41**, 391-400. (2008)

Fenech, M., N. Holland, E. Zeiger, W.P. Chang, S. Burgaz, P. Thomas, C. Bolognesi, S. Knasmueller, M. Kirsch-Volders and S. Bonassi: The HUMN and HUMNxL international collaboration projects on human micronucleus assays in lymphocytes and buccal cells past, present and future. *Mutagenesis*, **26**, 239-45 (2011)

Gadhave, P.D., R.S. Brar, H.S. Banga and A. Dhawan: λ- cyhalothrin induced genotoxicity in freshwater fish *Labeo rohita*. *Veterinary World*, **7**, 412-415.(2014)

Gnanavelrajah, N. and T. Kandasamy: Carbofuran adsorption, leaching and accumulation in edible tissues in three selected soils in tropics. American-eurasian. *J. Agricul. Environ. Sci*.,**12**, 1475-1478 (2012)

Guginski, G., A.P. Luiz, M.D. Silva, M. Massaro, D.F. Martins, J. Chaves, R.W. Mattos, D. Silveira, V.M.M. Ferreira, J.B. Calixto and A.R.S. Santos: Mechanisms involved in the antinociception caused by ethanolic extract obtained from the leaves of *Melissa officinalis* (lemon balm) in mice. *Pharmacol. Biochem. Behav*., **93**, 10-6 (2009)

Gupta, R.C.: Carbofuran toxicity. *J. Toxicol. Environ. Hlth*., **43**, 383-418 (1994)

Hamilton, M.A., R.C. Russo and R.V. Thurston: Trimmed Spearman-Karber method for estimating median lethal concentrations in bioassays. *Environ. Sci. Technol*.,**12**, 417-417 (1978)

Harabawy, A. S. A. and A. T. Ibrahim: Sublethal toxicity of carbofuran pesticide on the African catfish *Clarias gariepinus* (Burchell, 1822): Hematological, biochemical and cytogenetic response. *Ecotoxicol. Environ. Saf*.,**103**, 61-67, (2014)

Luzhna, L., P. Kathiria and O. Kovalchuk: Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. *Front. Genet*., **4**, 131. (2013)

Martins, E.N., N.T.C. Pessano, L. Leal, D.H. Roos, V. Folmer, G.O. Puntel, J.B.T. Rocha, M. Aschner, D.S. Ávila and R.L. Puntel: Protective effect of *Melissa officinalis* aqueous extract against Mn-induced oxidative stress in chronically exposed mice. *Brain Res. Bull*., **87**, 74-79 (2012)

Milatovic, D., R.C. Gupta and M. Aschner: Anticholinesterase toxicity and oxidative stress. *Sci. World J*., **6**, 295-310 (2006)

Mishra, K. and R.C. Sharma: Assessment of organochlorine pesticides in human milk and risk exposure to infants from North-East India. *Sci.Total Environ*., **409**, 4939-4949 (2011)

Nagaraja, P. and B.L. Bhaskara : Sensitive spectrophotometric assessment of carbofuran using dapsone as a new chromogenic reagent in formulations and environmental samples. *Eclética Química*, **31**, 43-48 (2006)

Naravaneni, R. and K. Jamil: Cytogenetic biomarkers of carbofuran toxicity utilizing human lymphocyte cultures *in vitro*. *Drug Chemical Toxicol*., **28**, 359-72. (2005)

Pandey, A.K., N.S. Nagpure and S.P. Trivedi: Evaluation of genotoxicity of profenofos to fresh water fish *Channa punctatus* (Bloch) using the micronucleus assay. African *J. Biotechnol*., **13** , 3985-3988

(2014)

Pickering, Q.H., C. Henderson and A.E. Lenke: The toxicity of organophosphorus insecticides to different species of warm water fishes. *Trans. Amer. Fish Soc*., **91**,175-184(1962)

Ruiz-Suárez, N., L.D. Boada, L.A. Henríquez-Hernández, F. González-Moreo, A. Suárez-Pérez, M. Camacho, M. Zumbado, M. Almeida-González, M. del Mar Travieso-Aja and O.P. Luzardo: Continued implication of the banned pesticides carbofuran and aldicarb in the poisoning of domestic and wild animals of the Canary Islands (Spain). *Sci. Total Environ*., **505**, 1093-1099 (2015)

Samarth, R.M., M. Panwar, M. Kumar and A. Kumar: Protective effects of *Mentha piperita* Linn on benzo[a]pyrene-induced lung carcinogenicity and mutagenicity in Swiss albino mice. *Mutagenesis*, **21**, 61-6 (2006)

Singh, R.K. and B. Sharma: Carbofuran-induced biochemical changes in *Clarias batrachus*. *Pestic. Sci*., **53**, 285-290 (1998)

Sowndhararajan, K. and S.C. Kang: Free radical scavenging activity from different extracts of leaves of *Bauhinia vahlii* Wight and Arn. *Saudi J. Biol. Sci*., **20**, 319-325 (2013)

Usmani, K.A., E. Hodgson and R.L. Rose: *In vitro* metabolism of carbofuran by human, mouse and rat cytochrome P450 and interactions with chlorpyrifos, testosterone and estradiol. *Chemico-Biological Interactions*, **150**, 221-32 (2004)

Vera-Candioti, J., S. Solonesk and M.L. Larramendy: Pirimicarb-based formulation induced genotoxicity and cytotoxicity in the freshwater fish *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, Poeciliidae). *Toxicol. Indus. Hlth*., **2013**, 1-10, (2013)

Waite, D.T., H. Sommerstad, R. Grover, L. Kerr and N.D. Westcott: Pesticides in ground water, surface water and spring runoff in a small saskatchewan watershed. *Environ. Toxicol. Chemi*., **11**, 741-748 (1992)

Wandan, E.N., M.J. Zabik and E.F. Elleingand: Determination of carbofuran in fish and corn leaves by two types of commercial immunoassays. *Scie. Res. Ess*., **6**, 1771-1779 (2011)

Wu, D. and A.I. Cederbaum: Alcohol, oxidative stress and free radical damage. *Alcohol Res. Hlth*., **27**, 277-84 (2003)

Zeljezic, D., A.L.Vrdoljak, N. Kopjar, B. Radicand and S. M. Kraus: Cholinesterase-inhibiting and genotoxic effects of acute carbofuran intoxication in man: a case report. *Basic Clin. Pharmacol. Toxicol*., **103**, 329-35(2008)

Zeljezić, D., A.L.Vrdoljak, B. Radić, N. Fuchs, S. Berend, V. Orescanin and N. Kopjar : Comparative evaluation of acetylcholinesterase status and genome damage in blood cells of industrial workers exposed to carbofuran. *Food Chem. Toxicol*., **45**, 2488-98 (2007).