MECHANISTIC STUDIES ON HEPATOTOXICITY OF THE NEONICOTINOID THIACLOPRID 21.7% SC IN SD RATS

INTRODUCTION
Thiacloprid [3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylidene cyanamide] is a member of the neonicotinoid insecticides registered for agricultural use by Bayer CropScience Ltd. (India) and is presently the most important commercial product because of its high efficacy against insects. It has outstanding potency and systemic action for crop protection against piercing-sucking pests, and is also highly effective for flea control on cats and dogs (Tomizawa and Casida, 2005).

Thiacloprid is reported selectively toxic to insects (Kagabu et al., 2002). All the neonicotinoids are synthesized by targeting the nicotinic acetylcholine receptor (nAChR) of crop insects. The neonicotinoids have unique physical and toxicological properties as compared with earlier classes of organic insecticides (Tomizawa and Casida, 2003). However, of the commercial neonicotinoids, acetamiprid, imidacloprid and thiacloprid are the most toxic to birds, and thiacloprid to fish. Several neonicotinoids are harmful to honeybees, either by direct contact or ingestion, but potential problems can be minimized or avoided by treating seeds and not spraying flowering crops (Kagabu and Akagi, 1997).

Upon uptake by mammals, most neonicotinoids undergo metabolic alterations at multiple sites but liver is a major site for metabolism of thiacloprid. The seven major commercial neonicotinoids are readily biodegraded by metabolic attack at their N-heterocyclylmethyl moiety, heterocyclic or acyclic spacer and N-nitroi mine, nitromethylene, or N-cyanoimine tip. Phase I metabolism is largely dependent on microsomal cytochrome P450 (CYP) isozymes with in situ selectivity in hydroxylation, desaturation, dealkylation, sulfoxidation and nitro reduction. Cytosolic aldehyde oxidase is a nitroreductase for some neonicotinoids. Phase II metabolism involves methylation, acetylation and formation of glucuronide, glucoside, amino acid and sulfate- and glutathione-derived conjugates. Some neonicotinoids act as proinsecticides, which get metabolized to more potent nicotinic agonists (Casida, 2011). During detoxification, thiazolidine ring of thiacloprid is opened and the sulfur oxidized and
methylated (Klein, 2001). The chloro substituent is displaced presumably by glutathione, ultimately leading (via cysteine and -SH derivatives) to a methylsulfide. The nitrosoguanidine metabolite of neonicotinoids has moderate to high toxic potency (Tomizawa et al., 2003), whereas the guanidine metabolite is highly activated against mammalian but deactivated against insect nAChRs (Chao and Casida, 1997; Tomizawa and Casida, 1999).

Cytochrome P450 is a superfamily of microsomal enzymes, which are found abundantly in the liver, gastrointestinal tract, lung and kidney, consisting of families and subfamilies of enzymes that are classified based on their amino acid sequence identities or similarities (Guengerich, 2003). Cytochrome P450 isozymes are involved in oxidative neonicotinoid metabolism especially the N-demethylated pathway (Schulz-Jander and Casida, 2002).

Moreover, thiacloprid undergoes CYP dependent oxidative metabolism to the N-demethylated products. The chloro substituent is displaced to form the methylsulfide. With dinotefuran, hydroxylation of the tetrahydrofuran moiety leads to ring opening and liberation of an aldehyde that forms cyclic derivatives (Tomizawa and Casida, 2005). The C=N-NO2 (nitroguanidine) moiety is reduced to C=N-NO (nitrosoguanidine) and C=N-NH2 (aminoguanidine) and cleaved to the C=NH (guanidine) and C=O (urea) derivatives (Figure A) (Tomizawa and Casida, 2005). Oxidative metabolism generates reactive oxygen species which

![Figure A. Hydroxylation, hydration, glucuronidation, and desaturation of Thiacloprid (THI) in vitro and in vivo (adapted from Tomizawa and Casida, 2005).](image-url)
are responsible for oxidative stress on liver. This may result in significant damage to cell structures (Devasagayam et al., 2004).

It is also known that free radicals play an important role in the toxicity of pesticides and environmental chemicals (D’Almeida et al., 1997). Pesticide chemicals such as insecticides may induce oxidative stress leading to generation of free radicals and alterations in antioxidants or free radical scavenging enzyme systems (Kanbur et al., 2008; Duzguner and Erdogan, 2010). The data from experimental models either in vivo or in vitro indicate that the enzymes associated with antioxidant defense mechanisms are altered under the influence of pesticides (Thapar et al., 2002; Singh et al., 2006). Moreover, oxidative stress and DNA damage have been proposed as mechanisms linking pesticide exposure to health effects such as cancer and neurological diseases. During the metabolism of insecticides, reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide (NO) could be generated (Grisham et al., 1999; Duzguner and Erdogan, 2012).

The elevated level of free radicals in liver leads to increased peroxidation of lipids and a decrease in the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase. Low concentration of these enzymes facilitates the hepatocytic damage of liver, the major site of metabolism. Also, transaminase and alkaline phosphatase are important and critical enzymes in the liver and are responsible for detoxification processes (Rahman et al., 2000). Hence, any interference in levels of these enzymes leads to biochemical impairment and lesions of the tissue. Serum activity of liver enzymes like transaminase, alkaline phosphatase and/or glutamate dehydrogenase have been reported to be altered due to the chronic exposure to this neonicotinoid (Bhardwaj et al., 2010; Kammon et al., 2010). Imidacloprid, a neonicotinoid, is a hepatotoxic pesticide which causes necrosis or hypertrophy (Mohany et al., 2011). Another study reported that during short term oral exposure to imidacloprid for 14 and 28 days, serum total protein, albumin, globulin and creatinine levels remained normal (Balani et al., 2011).

Altered activity of antioxidant system of hepatocytes and liver marker enzymes also reportedly reflects as a pathological change in the histoarchitecture of liver, the major detoxifying organ (Amacher, 2002). Histopathological investigation of rat liver after 10 days of exposure to another neonicotinoid, thiamethoxam, also reportedly disturbed hepatic lobules, causing hydropic degeneration in the hepatocytes and also induced dilation of the hepatic sinusoids.
Thiamethoxam treated rats showed vacuoles in the cytoplasm of the hepatocytes. Some liver cells showed pyknotic or karyolytic nuclei (Shalaby et al., 2010). Sub-chronic exposure of thiacloprid on Gallus domesticus in the experiment of Goyal et al. (2010) induced degeneration of hepatocytes, fatty changes along with vacuolation and focal necrosis of hepatocytes.

Thiacloprid, the test chemical being assessed in the current study, belongs to the neonicotinoid class of insecticides. It mainly targets the nervous system of insects by mimicking the neurotransmitter receptor of insects (EPA, 2005). Few studies have been performed related to the toxic effects of neonicotinoids in mammals. Moreover, these studies have mostly focused on the mechanism of neurotoxicity of neonicotinoids. However, many xenobiotics are capable of causing some degree of liver injury. The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation, and its anatomic and physiologic structure (Jones, 1996). Few studies have demonstrated that acute exposure to neonicotinoid imidacloprid leads to oxidative and inflammatory effects in rats (Duzguner and Erdogan, 2010). Although thiacloprid is the most frequently used insecticide amongst the pesticides, such studies assessing toxic potential of thiacloprid in mammals are insufficient. Studies elucidating the chronic effects the test substance in non-target organisms such as mammals can be of significance since it throws light on the safety evaluation of the compound. The objective of the current study therefore, was to find whether repeated oral administration of thiacloprid has the potential to induce toxicity in a mammalian model, the rat, with the prime focus on the liver, a major detoxification site.

In the present study, we investigated the potential oxidative and chronic inflammatory effects of thiacloprid on the hepatic system of rats. The study was designed to reveal whether subacute and subchronic oral exposures of thiacloprid can develop liver toxicity in rat. To fulfil this objective, changes in liver enzymes like the transaminases (ALT and AST), alkaline phosphatase, γ-glutamyl transpeptidase and lactate dehydrogenase were estimated. The potential oxidative damage, generated via metabolism of thiacloprid in liver was also investigated by estimating activity of anti-oxidative enzymes and non-enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidase, glutathione-S-transferase, catalase and reduced glutathione activity were quantified during the study. Estimation of lipid peroxidation product was also one of the parameters observed to assess oxidative damage. The potential
relation between alterations in liver enzyme activity or antioxidant enzyme activity with histoarchitecture of liver was also explored.

**MATERIAL AND METHODS**

Thirty male Sprague Dawley (SD) rats weighing 250 ± 20 gms were used for the present study. The animals were kept in clean cages in a well-ventilated animal house of the Department of Zoology at The M.S. University of Baroda, Vadodara. All the protocols for experiments were approved by IAEC of the department of Zoology according to CPCSEA, India. Animals were exposed to a 12:12 light-dark schedule and were provided with standard rat feed (Pranav Agrochemicals, India) and RO water *ad libitum*. They were acclimatized for 10 days before starting the experimental procedure. Thiacloprid (Alanto 240, 21.7% SC) was diluted in physiological saline to obtain the desired dose concentrations. Animals were divided into following three groups of 5 animals each: Group 1, Control; Group 2, low dose [50 mg/kg body weight thiacloprid daily dose, (LTD)]; Group 3, High dose [100 mg/Kg/body weight thiacloprid daily dose (HTD)] with 15 rats used for the 28 days study and 15 rats for the 90 days study. The rationale for selecting the said doses has been discussed elsewhere (Material and Methods). Food consumption was noted everyday and animals were weighed every week for analysis of body weight.

The animals were sacrificed 24 hours after the last drug administration. Before sacrifice, animals were subjected to overnight fasting and blood was collected from the orbital sinus of rats. They were sacrificed by cervical dislocation under mild diethyl ether anaesthesia. Liver was quickly excised from each rat, washed with ice-cold phosphate buffer saline, blotted free of fluids and used for biochemical studies. A part from the right lobe of liver was also fixed in 10% neutral buffered formalin for histopathological study. Blood was kept at 4°C for 2 hrs and serum was separated after centrifugation. Tissue samples were homogenized in ice cold phosphate buffer and centrifuged at 4°C on 3000 rpm for 15 minutes. Supernatant was removed and used for biochemical studies.

**Protocol I: Biochemical estimation**

1. **Assessment of enzyme activity**

Alanine transaminase (ALT) (E.C. 2.6.1.2) was estimated by the method of the International federation of clinical chemistry (IFCC) (Bergmeyer and Horder, 1980). The ALT activity in
the sample is proportional to the decrease in rate of absorbance of NADH which is measured at 340nm kinetically for 2 minutes. Aspartate transaminase (AST) (E.C. 2.6.1.1) level was estimated by the method of the IFCC (Bergmeyer et al., 1978). The AST activity in the sample is proportional to the decrease in rate of absorbance of NADH which was measured at 340nm kinetically for 2 minutes.

Activity of alkaline phosphatase (ALP) (E.C. 3.1.3.1 was estimated by the protocol of Tietz (1983) approved by IFCC. The activity of the alkaline phosphatase was measured colourimetrically at 410nm. γ-glutamyl transpeptidase (GGT) (E.C. 2.3.2.2) activity was measured at 405nm according to calibrated method of Szasz (1969). Lactate dehydrogenase (LDH) (E.C. 1.1.1.27) activity was measured as per the protocol of Buhl et al. (1977) at 340nm.

2. Estimation of biomolecule

Total protein concentration was measured at 660nm according to the method of Lowry et al. (1951). Glucose level was estimated using GOD/POD (Trinder, 1969) method and absorbance was measured at 505nm.

Total bilirubin (metabolite) level in serum was measured using the method described by Jendrassik and Grof (1938). The intensity of colour produced in reaction is proportional to the concentration of bilirubin and was measured at 546nm.

Protocol 2: Oxidative stress parameters

The lipid peroxidation product present in the tissues was estimated by thiobarbituric acid (TBA) method (Janero, 1998). Red coloured complex of TBA reactive substance (TBRS) was measured at 532nm colourimetrically.

Catalase (E.C. 1.11.1.6) activity was assayed by the method of Sinha et al. (1972). Marklund and Marklund (1974) method was used for assessment of superoxide dismutase (SOD) (E.C. 1.15.1.1) activity. SOD inhibits the auto oxidation of pyragallol in a rate limiting fashion which was assessed at 420nm. The activity of glutathione peroxidase (GPx, E.C. 1.11.1.9) in sample was determined by the method of Rotruck et al. (1973). Enzyme preparation was allowed to react with H2O2 in presence of GSH for a specific time period. The GSH content remaining after the reaction was measured by the method of Ellman et al. (1961).

Habig et al. (1994) method was adopted for estimation of glutathione-S-transferase (GST, E.C. 2.5.1.18) activity which was measured by following the increase in absorbance at 340nm using 1-Chloro-2,4-dinitrobenzene (CDNB) as a substrate.
The level of reduced glutathione, a non enzymatic antioxidant, was determined by the method of Beutler et al. (1963). This method is based on the development of yellow colour when thiol reagent, 5,5’-dithio-bis-2-nitrobenzoic acid (DTNB) reacts with GSH present in tissue sample forming 5-Thionitrobenzoic acid (TNB) and GS-TNB, which can be measured at 412nm. The level of TNB and GS-TNB is equivalent to the GSH present in the tissue.

Protocol 3: Histopathological Examination
After overnight fasting, rats were sacrificed and livers were dissected out immediately. For the histopathological evaluation of liver, tissue was first fixed in 10% neutral buffered formalin solution for 24 hours and dehydrated in an upgrading ethanol series. Xylene was used as clearing agent before embedding in paraffin wax. Paraffin blocks of tissues were sectioned using a microtome, rehydrated and then stained with haematoxylin and eosin. Sections were dehydrated again, following which they were mounted in DPX. Slides were observed under the microscope (Leica DM2500) for analysis.

Statistical analysis
Statistical analysis was carried out using SPSS 12.0 for Windows and GraphPad Prism (version 6). The significance of the differences between mean values was evaluated through one-way ANOVA. Post hoc comparisons were carried out using the Bonferroni test. The data are presented as mean ± SEM. The minimum level of significance was set at p ≤ 0.05.

RESULTS
In the current oral exposure study, rats were subjected to 50 and 100mg/kg body weight of thiacloprid (daily) and conditions such as bradypnea, laboured breathing, tremors, reduced motility and nose bleeding were observed in these animals during the period of exposure. After 26 days of thiacloprid exposure, clear ptosis was observed in rats receiving high dose and after 40 days, ptosis was common in both the treatment groups. Thiacloprid exposure for one month reduced the hind leg movement and it was observed that animals developed a drag during movement. This observation was common for both the treatment groups after 40 days of exposure to the test compound. Also, nasal secretion was commonly observed. Piloerection of hair situated around the eyes was observed routinely after exposure. Further, during subchronic exposure to thiacloprid, two rats from high dose group showed signs of extreme intoxication.
viz., difficulty in locomotion, excessively laboured breathing, and were also unable to consume food and water after 78 days and had to be sacrificed early.

The weekly food intake of control and experimental groups of rats is given in Table 1.1. Food consumption in treatment groups was slightly lower during the toxicity period of evaluation of thiacloprid in the 28 days study as compared to control group (Figure 1.1). Values in Table 1.2 show significantly lower food consumption by rats during the 90 days treatment period, both for the low dose ($p \leq 0.05$) and high dose ($p \leq 0.01$) groups (Figure 1.2).

The mean alterations in body weight of rats are summarized in Table 1.3. For animals of both high and low dose treatment groups, a decrease in body weight was observed during the 28 days study, but values were not significantly different from those of control animals. However, significant decrease in the body weight of both low dose ($p \leq 0.05$) and high dose ($p \leq 0.01$) treatment groups of animals was observed during the subchronic study.

At the end of the drug exposure period, rats were sacrificed and observations on gross anatomy were also made. ‘Milk spots’ described as white patched lesions of necrosis (Biehl, 1984) were commonly observed in animals of the high dose treatment group during both the studies and also in animals of high dose group during subacute study. Also, a change in spleen colour and necrosis at its edges was observed in high dose group. White cysts of necrosis were also observed. Table 1.3 shows liver weight at the end of treatment schedule for the subacute and subchronic studies. Weight of liver was found to be increased significantly ($p \leq 0.05$) during subchronic exposure at 100mg/kg body weight as compared to that of control group of rats (Figure 1.3).

The effect of repeated 28 day exposure to thiacloprid at doses of 50mg/kg and 100mg/kg/body weight on serum enzyme parameters is shown in Table 1.4 and Figure 1.4. Alanine aminotransferase activity increased significantly ($p \leq 0.05$) in serum of high dose treated rats as compared to controls. However, difference in ALT activity between low dose and control group was marginal and was not statistically significant during this treatment regime. Aspartate transaminase activity increased insignificantly in serum of both groups of treated rats as compared to control rats. It was observed that exposure to both 50mg/kg and 100mg/kg body weight of thiacloprid caused significant increase in alkaline phosphatase activity. Level of significance was low ($p \leq 0.05$) for LTD and high ($p \leq 0.01$) for HTD group of rats. Neither
dose group of thiacloprid treatment showed any significant change on γ-glutamate transpeptidase (GGT) activity. Lactate dehydrogenase activity was not significantly altered in low dose group but was seen significantly increased (p ≤ 0.05) in high dose treatment group as compared to values of control group of animals.

Activities of various liver marker enzymes following subchronic thiacloprid exposure are shown in Table 1.5 and Figure 1.5. The obtained results indicate that 1/15 and 1/30 of median lethal dose of thiacloprid induced significant increase in ALT activity (p ≤ 0.05) for both low and high dose groups (p ≤ 0.001) of rats. Activity of ALT was observed to be significantly (p ≤ 0.05) higher in HTD as compared to low dose treatment group. AST activity was found to be increased for the low dose group as compared to control but the difference was not found statistically significant. AST activity was observed to be significantly higher (p ≤ 0.05) in 100mg/kg body weight thiacloprid treatment group. Oral administration of test chemical induced hepatic damage by altering the activity of ALP. Significantly higher ALP activity was observed in both low dose (p ≤ 0.05) and high dose (p ≤ 0.001) groups as compared to control group. GGT activity in serum was observed higher in both the treated groups of rats as compared to control group of animals. In both the treatment groups, significant increase in level of LDH enzyme activity was also observed. As compared to control group, the activity of LDH was higher at p ≤ 0.05 for LTD and p ≤ 0.01 for HTD rats.

Mean alterations in biomolecule concentration in serum for both subacute and subchronic studies are summarized in Table 1.6 and 1.7 respectively. At the end of 28 days of thiacloprid exposure, there was no significant difference in total serum protein concentration between the treatment groups of rats. Even though the treated animals showed marginal hike in serum protein concentration compared to controls they were statistically not significant. Protein concentration in serum was significantly higher (p ≤ 0.05) after subchronic exposure to thiacloprid in both the treatment groups as compared to control group of rats. Glucose concentration was observed to be drastically low in both the low dose (p ≤ 0.01) and high dose (p ≤ 0.001) groups after subacute oral exposure to thiacloprid (Figure 1.7). Glucose concentration decreased significantly (p ≤ 0.001) in serum of both the treatment groups due to subchronic exposure to thiacloprid. Subacute and subchronic exposure to 50mg/kg and 100mg/kg body weight doses of thiacloprid did not cause any significant change in serum bilirubin concentration for the treated rats as compared to control rats. At the end of 90 days of exposure of test chemical, marginal decrease in total bilirubin was observed (Figure 1.6).
Table 1.8 summarizes the effect of thiacloprid on LPO activity and antioxidant enzyme activity in liver of rats after 28 days of administration as compared with control. The liver lipid peroxide levels were high in the case of treated animals. LPO activity in terms of MDA level was found significantly increased by 100mg/kg body weight administration of thiacloprid (p ≤ 0.05). Similarly a higher level of liver lipid peroxide in low dose treatment group was observed but, difference was not found statistically significant. Liver catalase level was found to be depleted significantly in HTD group of rats as compared to control group of rats (p ≤ 0.01). Non significant change was found by administration of 50mg/kg body weight thiacloprid for 28 days. Liver superoxide dismutase activity was not found to be altered significantly after administration of thiacloprid in both treatment groups. Glutathione peroxidase activity was found elevated in both the treatment groups as compared to that of control rats, but values were not statistically significant. Activity of glutathione-S-transferase in HTD group was observed to be higher, but not statistically significant as compared to control group of rats. GST activity in LTD treatment group was not much different compared to control group of animals. Decreased level of reduced glutathione was observed in both the treatment groups as compared to control group, but the decrease was significant (p ≤ 0.05) only for the high dose group (Figure 1.8).

Table 1.9 represents the subchronic effect of thiacloprid on liver LPO and stress marker enzyme activity. A statistically higher activity of LPO was found in both the treatment groups of rats (p ≤ 0.05) as compared to control rats. Liver catalase level was found to be decreased in thiacloprid exposed rats as compared to control rats and activity was significantly lower in high dose group (p ≤ 0.01). The low level of liver SOD in treated animals was found significant (p ≤ 0.05) in HTD group and non significant in LTD group compared to reference group of rats. Lowered activity of GPx was found to be statistically significant in the 100mg/kg body weight treatment group as compared to control. GST activity was also observed to be lower in treatment group of rats with difference statistically significant for the HTD group (p ≤ 0.05). Non enzymatic antioxidant level was also found to be depleted after subchronic intubation of thiacloprid with significantly low values for both LTD (p ≤ 0.05) and HTD (p ≤ 0.01) groups as compared to control group (Figure 1.9).

**Histopathological changes in liver**

Microscopic examination of liver of control rats showed normal structure of the central vein, radially arranged hepatocytes around the central vein and blood sinusoids (Figure 1.10 and
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1.1. Histopathological investigation of liver sections of the treated rats showed disturbed hepatic lobule with variable degrees of hepatic degeneration in many areas of liver. Vacuolar hepatocyte degeneration and loss of radiating hepatocyte arrangement were very apparent (Figure 1.12 to 1.15). Centrilobular hepatic damage (zonal necrosis) and focal area necrosis were observed in treated liver (Figure 1.16). Infiltration of inflammatory cell was also seen (Figure 1.17).

DISCUSSION
Thiacloprid is a novel neonicotinoid insecticide belonging to sub class of nicotinyl compounds. It is used as a systemic insecticide for soil and foliar applications to control a variety of insect pests (EPA, 2003). The current study was designed to assess the toxicity potential of this pesticide on mammals, which are the non-target organisms, using SD rat as the animal model. Results of this study point towards significant incidence of toxicity in SD rats due to subacute and subchronic exposure to thiacloprid. These included nose bleeding, laboured breathing, reduced motility, ptosis and tremors. It was found that exposure to high dose of thiacloprid resulted in higher and cumulative toxic effects than the lower dose indicating a dose dependent toxic response towards the pesticide. Kammon (2010) had reported similar observations in layer chicken on exposure to another variant of neonicotinoid imidacloprid, providing support to the results of the present study. A decrease in body weight gain was also observed in animals exposed to thiacloprid daily at a higher dose of 100 mg/kg/body weight during the 90 days study period. In contrast, no change in the body weight was observed during subacute treatment of thiacloprid. Similar findings have been mentioned in the study reports of EPA (2003) and JMPR (2010), wherein a decrease of body weight gain was reported in male rats given 60mg/kg/day and 120mg/kg/day dose of thiacloprid.

The amount of food intake was also measured every day during the period of study. Lower food consumption by rats in the treatment group was observed during the subchronic study and this can be correlated to the earlier observation of reduced body weight in the treated animals. Observations made by Goyal (2010) during a toxicity study of thiacloprid in the digestive tract of birds, indicate that thiacloprid act as an irritant to the intestinal membrane, and opined that this could reduce food consumption. A similar response of the thiacloprid in rat intestine cannot be ruled out and might have resulted in reduced food intake in animals subjected to subchronic treatment. Hence, in the current study, overall low motility, general weakness and
low food intake could be the reasons for observed weight loss in animals exposed to thiacloprid during the treatment period.

Liver weight of thiacloprid treated rats was found to be increased during subchronic study. Liver enlargement can occur as a result of changes in dietary composition or metabolic aberration Liver enlargement without the accompanying histopathological change or functional impairment is often interpreted as being a physiological adaptation to enhanced workload or metabolic demand in body (Chopra and Griffin, 1985). The enlargement of the liver in the present study was probably due to the functional hypertrophy of the smooth endoplasmic reticulum and increased drug metabolizing multi-enzyme complex, as suggested by Zimmerman (1999). The reduced food consumption and increased liver weight in high dose exposed rats might be due to the toxic potential of neonicotinoid. White patches and necrotic bodies were commonly observed as an anatomical change in liver of treated male rats. This prominent necrosis in liver might be due to the transport of some toxic metabolite from intestine to liver and the presence of definite necrosis indicates towards the capability of the toxic metabolite in causing cell death. Similar anatomical changes in liver have also been observed by Shalaby (2010) in albino rats exposed to thiamethoxam, by Omima (2004) in Japanese quail received imidacloprid and also by Kammon (2010) in layer chicken subjected to neonicotinoid imidaclorpid.

Activities of serum enzymes like AST, ALT and ALP represent the functional status of liver (Mohany et al., 2011). High serum levels of AST and ALT are usually indicative of liver damage in animals (Durak et al., 1996) and humans (Ray and Drummond, 1991). In this study, there was a significant increase in the activity of serum enzymes, namely, ALT and AST in rats given orally the thiacloprid at the high dose of 100mg/kg body weight during both subacute and subchronic exposure regime. The level of ALT was also significantly higher in low dose treated rats (50mg/kg body weight) during the subchronic study. Freedland and Kramer (1970) suggested that enzyme levels are sensitive indicators of tissue damage, since they are liberated from cells even when the magnitude of lesions is not sufficient for morphological detection. Increased enzyme activity seen in the current study probably could be due to prolonged exposure to thiacloprid as a mechanism employed for detoxification of this pesticide. Increased enzyme activity may possibly be based on mutation of genes for the synthesis of these enzymes (Bolognesi and Morasso, 2000). Balani et al. (2011) who studied toxicity in male white leghorn chicks treated with different concentrations of neonicotinoid...
imidacloprid have also reported an elevated level of transaminase enzyme. Shakoori et al. (1994) reported that the increase in plasma ALT activity is mainly due to the leakage of this enzyme from the liver cytosol into the blood. Further, alkaline phosphatase activity was also observed to be elevated in both the treatment groups during both subacute and subchronic studies.

Exposure to neonicotinoid imidacloprid reportedly induces hepatocyte damage and increases ALP, AST and ALT activity in serum of animals (Bhardwaj et al., 2010; Aydin, 2011; Toor et al., 2012). The metabolites of thiacloprid produced during its biotransformation may also be responsible for hepatotoxicity and the increase in serum activities of liver enzymes. Liver-ALP is mobilized most rapidly into blood and its levels in serum may increase at early period of liver damage. High ALP serum level is usually indicative of cholestasis which may also result in progressive liver disease like biliary cirrhosis (Alvaro et al., 2000). In the present study also similar increase in serum levels of all parameters have been observed, indicative of liver damage due to exposure to neonicotinoid thiacloprid.

In the current investigation, GGT activity was also found elevated in rats at 90 days of exposure to thiacloprid. GGT activity is a more sensitive marker for cholestatic damage than ALP and is very specific to the liver ( McClatchey, 2002). Another biochemical marker used to evaluate liver function is lactate dehydrogenase (LDH) activity which was found to be increased in serum of thiacloprid treated groups of rats in present study. Increased LDH activity possibly indicates towards liver damage and it may be attributed to a generalized increase in membrane permeability, as reported by Kaczor et al. (2005). Moreover, elevation of LDH activity also indicates towards cell lysis and death as well as towards the switching over of aerobic glycolysis to anaerobic respiration. The increased serum LDH activity may be due to hepatocellular necrosis leading to leakage of the enzyme and enzyme inhibition in liver which is supported by decrease in soluble protein (Shakoori et al., 1994). LDH can be used as an indicator of the potential of toxic agents to cause cellular damage (Bagchi et al., 1995). Similar results of increased LDH activity have been reported by Zaahkook et al. (2009) in Japanese quail after 3 weeks of imidaclorpid exposure. Except for this study, literature concerning GGT and LDH activity after exposure to thiacloprid and any other neonicotinoid insecticides are lacking.
The total serum protein content was found to be higher in the rats exposed to different concentrations of the thiacloprid than the control during the 28 days study, but the difference was not statistically significant. However, significantly higher concentration of serum protein was observed in the treated rats after 90 days of thiacloprid exposure. A similar result has been given in EPA fact sheet (2003), where protein level increased after 6 weeks of thiacloprid exposure. Elevation in total protein content may be due to the hepatic detoxification, which results in the inhibitory effect on the activities of enzymes involved in detoxification. However, researchers have also reported a reduction in rat plasma protein due to exposure to different concentrations of neonicotinoid imidacloprid (Zaahkook et al., 2009; Aydin, 2011). Significantly decreased serum glucose level was observed for the treated rats during both the studies. This observation finds support from a similar finding available in NRA (2001) public release summary and EPA (2003). Other such comparable reports on effects of thiacloprid on glucose levels are not available. Hypothetically, the low glucose level in the treated rats can be correlated to the low food intake in these animals as compared to the control group or it may be possible that thiacloprid affects the process of glucose absorption. Subacute exposure to thiacloprid did not cause any significant alteration in serum total bilirubin in the current investigation. However, subchronic oral administration of test compound decreased total bilirubin concentration in serum of rats. NRA (2001) public summary by Australia reported a similar finding but other research data regarding effects of this test compound on bilirubin are not available. However, Bhardwaj et al. (2010) have made a similar observation regarding bilirubin during a study conducted using the neonicotinoid imidacloprid on female rats.

Once thiacloprid enters in the biological system, it is transformed into primary and secondary metabolites. These intermediary metabolites, responsible for the hepatotoxic effect of test compound, may bind to cellular macromolecules and react with free amino groups of proteins; hence, the macromolecules may lose their physiological functions (Teppema et al., 2002) or stimulate hepatocytes to produce more toxic metabolites (Durak et al., 1996). They may cause cellular damage by covalent binding to cellular components such as enzymes, nucleic acids, and proteins or by any another mechanisms. Damage of cellular components may play an important role in death of liver cells (Fee et al., 1979; Teppema et al., 2002) and increased oxidative stress. Consequently, serum levels of AST, ALT, and ALP enzymes may increase. Besides, increased levels of liver LDH may result due to superoxide anions and hydroxyl radicals which cause oxidative damage to the cell membrane (Yadav et al., 1997). Elevated free radicals and depressed antioxidant defense may lead to cell disruption, oxidative damage
to cell membrane and hence, increase susceptibility to lipid peroxidation (Kapoor et al., 2009; Kapoor et al., 2010). Elevated level of MDA, a product of lipid peroxidation (LPO), suggests an increased production of free oxygen radicals in rats (Mansour and Mossa, 2009). In the present work, thiacloprid significantly induced LPO and decreased other vital antioxidant enzymes in liver at high dose during subacute study and at both the doses during subchronic study. Susceptibility of liver to this stress due to exposure of thiacloprid is a function of overall balance between degree of oxidative stress and antioxidant capacity (Khan et al., 2005). High activity of LPO in liver in our study suggested the production of oxidative metabolites or free radicals during hepatic metabolism and this may be due to the progressive nature of free radical chain reaction. Increased level of MDA in liver tissues in the present study can be ably supported with similar results reported by Bhardwaj et al. (2010), Kapoor et al. (2010) and Aydin (2011) from studies conducted in rats using imidacloprid.

Oxygen free radicals and hydroperoxide collectively termed as reactive oxygen species (ROS) are produced by univalent reduction of dioxygen to superoxide anion (O$_2^-$) which in turn is converted into H$_2$O$_2$ and O$_2$ through a reaction catalyzed by SOD (Rai and Sharma, 2007). Antioxidant enzymes (SOD and Catalase) constitute the first line of defense against deleterious effect of oxyradicals in cells by catalyzing dismutation of superoxide radical. The observed decrease in SOD activity in liver of thiacloprid intoxicated rats during 90 days of exposure may be due to the consumption of this enzyme during conversion of O$_2$ to H$_2$O$_2$. Similar decreased activity of SOD was also reported with imidacloprid pesticide in rats (Kapoor, 2010; Duzguner and Erdogan, 2012). Finally, increased H$_2$O$_2$ resulting from catalase inhibition reduces SOD activity (Yu, 1994) in tissue. The current results were in accordance to these reports.

Various studies have demonstrated decrease in GPx activity due to xenobiotics (Kapoor et al., 2009; Mansoor and Mossa, 2009; Kapoor et al., 2010). Similar findings have been observed for the high dose treatment group during the present subchronic investigation. GSH plays a key role in the modulation of pesticide induced oxidative damage in tissues. GSH depletion evidently intensifies LPO and is known to predispose cells to oxidative damage (Khan et al., 2005). A significant depletion of GSH in liver in animals of both treatment groups during subchronic study and high dose group during subacute exposure together with decrease in activity of GPx may induce oxidative damage in liver tissue of rats. In addition, GSH participates in detoxification of xenobiotics as substrate for enzyme Glutathione-S-transferase
(GST) which is found to be reduced by thiacloprid in present investigation, which may be due to direct utilization of GSH as an antioxidant in terminating free radical reaction. Similar reduction in GSH has been reported earlier in rats with imidacloprid exposed liver as target organ (Gendy et al., 2010; Kapoor et al; 2010)

The decreased activities of SOD, GPx, Catalase and GSH together with increased LPO may have led to free radical toxicity during subchronic exposure to thiacloprid. Thiacloprid toxicity may also induce histopathological alterations in liver.

The increase in activities of specific liver enzymes and LPO activity correlate well with the gross and histopathological changes in treated liver observed in the present investigation. Increase in enzyme activity in serum may be due to enzyme loss in liver tissue (Bhardwaj et al., 2010). Histopathological findings also support the gross anatomical changes like white necrotic patches and necrotic cyst observed as an anatomical change at the time of sacrifice. Goyal et al. (2010) observed marked degeneration of hepatocytes in thiacloprid exposed Gallus domesticus and also observed changes such as vacuolation and focal necrosis of hepatocytes. Similar changes have also been observed during the current study. The present information gathered on liver changes induced by thiacloprid is both limited and comparable with another neonicotinoid the imidacloprid (Bhardwaj et al., 2010; Kammon et al., 2010; Toor et al., 2012). Leucocytic infiltration was also observed by Toor et al. (2012) in female rats exposed to imidacloprid. Cyano group containing neonicotinoid thiamethoxam is also known to induce hepatic degeneration in liver tissue of albino rats (Shalaby et al., 2010).

CONCLUSION
The result of present study revealed that thiacloprid at low dose induces only subtle hepatotoxicity in rat when exposed for a short duration. However, subchronic exposure to thiacloprid significantly induced hepatic damage as evident from the increased levels ALT, AST, LDH and ALP enzyme activity and by generating oxidative stress. This may be due to disturbed cellular oxidative status as evidenced by increased LPO activity, decreased activities of SOD, catalase, GST, GPx and reduced GSH level in liver. Increased oxidative stress might have led to the histopathological changes observed in the study. Vacuolar hepatocyte degeneration and centrilobular necrosis were common histopathological changes observed in liver tissue which supports our presently observed deranged biochemical profile in the liver of thiacloprid exposed rats. Moreover, decreased food intake and increased liver weight in the
treatment groups may also be the result of thiacloprid intoxication. Thus, based on the results of the current study, it can be concluded that long term exposure to thiacloprid can potentially induce moderate to severe hepatic damage in the mammalian system.
Table 1.1. Weekly food consumption by rats per day during 28 days oral thiacloprid exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0 gm/day</th>
<th>Day 7 gm/day</th>
<th>Day 14 gm/day</th>
<th>Day 21 gm/day</th>
<th>Day 28 gm/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.3±0.96@</td>
<td>11.7±1.2</td>
<td>11.6±1.56</td>
<td>12.6±1.6</td>
<td>12.8±1.47</td>
</tr>
<tr>
<td>Low Dose</td>
<td>10.9±1.5</td>
<td>10.6±1.8</td>
<td>11.42±0.9</td>
<td>11.7±1.59</td>
<td>12.43±1.24</td>
</tr>
<tr>
<td>High Dose</td>
<td>11.58±1.1</td>
<td>11.16±1.6</td>
<td>11.14±1.22</td>
<td>11.69±1.14</td>
<td>11.81±1.82</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE; n=5 for each group.

Table 1.2. Monthly food consumption by rats during subchronic thiacloprid oral exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>One month gm/day</th>
<th>Second month gm/day</th>
<th>Third month gm/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12±1.6*</td>
<td>14.95±0.98</td>
<td>16.53±1.44</td>
</tr>
<tr>
<td>Low dose</td>
<td>11.65±1.23</td>
<td>12.34±1.5</td>
<td>10.32±1.56*</td>
</tr>
<tr>
<td>High dose</td>
<td>11.46±1.42</td>
<td>10.42±1.87↓*</td>
<td>7.85±1.39↓**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE; n=5 for each group; * p ≤ 0.05; ** p ≤ 0.01

Table 1.3. Effect of thiacloprid on animal weight and absolute liver weight after subacute and subchronic exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>Subacute</th>
<th>Subchronic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animal weight (gm)</td>
<td>Organ weight(gm)</td>
</tr>
<tr>
<td>Control</td>
<td>280±8.9*</td>
<td>12.3±0.87</td>
</tr>
<tr>
<td>Low dose</td>
<td>278±8.99</td>
<td>12.19±1.2</td>
</tr>
<tr>
<td>High dose</td>
<td>278.65±10.6</td>
<td>13.2±1.56</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE; n=5 for each group; * p ≤ 0.05; ** p ≤ 0.01

Table 1.4. Effect of thiacloprid on biochemical markers of liver function: Enzyme activities in the serum of control and treated SD rats after 28 days of exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT IU/L</th>
<th>AST IU/L</th>
<th>ALP μM pNP release/mg tissue</th>
<th>GGT IU/L</th>
<th>LDH IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.7±0.88*</td>
<td>10.9±0.07</td>
<td>83.1±1.8</td>
<td>4.5±0.37</td>
<td>37.8±0.7</td>
</tr>
<tr>
<td>Low dose</td>
<td>36.4±0.87</td>
<td>11.8±0.51</td>
<td>94.5±2.3↑*</td>
<td>4.5±0.51</td>
<td>37.9±0.71</td>
</tr>
<tr>
<td>High dose</td>
<td>39.1±1.2↑*</td>
<td>12.5±0.63</td>
<td>99.3±3.3↑**</td>
<td>5.0±0.72</td>
<td>40.9±0.77↑*</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE; n=5 for each group; * p ≤ 0.05; ** p ≤ 0.01
Table 1.5. Effect of thiacloprid on biochemical markers of liver function: Enzyme activities in the serum of control and treated SD rats after 90 days of exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT IU/L</th>
<th>AST IU/L</th>
<th>ALP μM pNP release /mg tissue</th>
<th>GGT IU/L</th>
<th>LDH IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.2±1.13*</td>
<td>10.1±0.36</td>
<td>87.5±1.8</td>
<td>6.06±0.29</td>
<td>37.5±0.58</td>
</tr>
<tr>
<td>Low dose</td>
<td>42.1±1.16↑*</td>
<td>12.4±0.98</td>
<td>96.8±1.7↑*</td>
<td>7.99±0.35↑*</td>
<td>41.1±0.57↑*</td>
</tr>
<tr>
<td>High dose</td>
<td>46.7±1.1↑***a</td>
<td>13.8±0.62↑*</td>
<td>105.7±2.4↑***a</td>
<td>7.98±0.49↑*</td>
<td>42.4±0.82↑**</td>
</tr>
</tbody>
</table>

*Values are expressed as Mean±SE; n=5 for each group; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001;  a significantly higher than low dose (a p ≤ 0.05)

Table 1.6. Effect of subacute thiacloprid intoxication on serum biochemical parameters in SD rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein gm/dl</th>
<th>Glucose mg/dl</th>
<th>Bilirubin mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.7±0.29***</td>
<td>132.7±3.4</td>
<td>1.45±0.099</td>
</tr>
<tr>
<td>Low dose</td>
<td>5.2±0.35</td>
<td>105.4±4.8↑**</td>
<td>1.47±0.10</td>
</tr>
<tr>
<td>High dose</td>
<td>5.6±0.33</td>
<td>98.73±5.1↓***</td>
<td>1.47±0.11</td>
</tr>
</tbody>
</table>

*Values are expressed as Mean ± SE; n=5 for each group; ** p ≤ 0.01; *** p ≤ 0.001

Table 1.7. Effect of subchronic thiacloprid intoxication on serum biochemical parameters in SD rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein gm/dl</th>
<th>Glucose mg/dl</th>
<th>Bilirubin mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7±0.15***</td>
<td>119.8±2.7</td>
<td>1.55±0.06</td>
</tr>
<tr>
<td>Low dose</td>
<td>8.2±0.47↑*</td>
<td>87.7±4.98↓***</td>
<td>1.45±0.12</td>
</tr>
<tr>
<td>High dose</td>
<td>7.99±0.77↑*</td>
<td>84.5±4.88↓***</td>
<td>1.37±0.10</td>
</tr>
</tbody>
</table>

*Values are expressed as Mean ± SE; n=5 for each group; * p ≤ 0.05; *** p ≤ 0.001
Table 1.8. Effect of thiacloprid on the activities of LPO, antioxidant enzyme and non enzymatic antioxidant after sub acute oral exposure in rat liver

<table>
<thead>
<tr>
<th>Group</th>
<th>LPO (nmol/min/mg tissue)</th>
<th>Catalase µmole H₂O₂ liberate/ minute/ mg protein</th>
<th>SOD (% inhibition /min/mg tissue)</th>
<th>GST µmoles of GSH / minute/ mg protein</th>
<th>GPx (mM of GSH consumed/ mg tissue)</th>
<th>GSH (µg/ gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.9±0.77@</td>
<td>63.1±2.3</td>
<td>11±0.32</td>
<td>4.7±0.43</td>
<td>10.6±0.51</td>
<td>10.7±0.13</td>
</tr>
<tr>
<td>Low dose</td>
<td>21.5±1.06</td>
<td>66.3±2.2</td>
<td>10±0.43</td>
<td>4.6±0.59</td>
<td>12.8±0.98</td>
<td>9.5±0.35</td>
</tr>
<tr>
<td>High dose</td>
<td>22.1±1.22↑*</td>
<td>79.3±2.8↑**</td>
<td>11.6±0.47</td>
<td>6.3±0.63</td>
<td>12.7±1.1</td>
<td>8.7±0.41↑*</td>
</tr>
</tbody>
</table>

@Values are expressed as Mean ± SE; n=5 for each group; * p ≤ 0.05; ** p ≤ 0.01

Table 1.9. Effect of thiacloprid on the activities of LPO, antioxidant enzyme and non enzymatic antioxidant after subchronic oral exposure in rat liver

<table>
<thead>
<tr>
<th>Group</th>
<th>LPO (nmol/min/mg tissue)</th>
<th>Catalase µmole H₂O₂ liberate/ minute/ mg protein</th>
<th>SOD (% inhibition /min/mg tissue)</th>
<th>GST µmoles of GSH / minute/ mg protein</th>
<th>GPx (mM of GSH consumed/ mg tissue)</th>
<th>GSH (µg/ gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.3±0.7*</td>
<td>27.9±0.8</td>
<td>14.7±0.54</td>
<td>5.4±0.28</td>
<td>12.6±0.37</td>
<td>10.2±0.36</td>
</tr>
<tr>
<td>Low dose</td>
<td>30.8±0.9↑*</td>
<td>25.5±0.9</td>
<td>13.7±0.45</td>
<td>4.5±0.57</td>
<td>10.9±0.54</td>
<td>7.98±0.54↑*</td>
</tr>
<tr>
<td>High dose</td>
<td>31.8±1.6↑*</td>
<td>22.5±1.1↓**</td>
<td>12.3±0.42↓*</td>
<td>3.5±0.31↓*</td>
<td>10.8±0.46↓*</td>
<td>7.29±0.39↓**</td>
</tr>
</tbody>
</table>

*Values are expressed as Mean ± SE; n=5 for each group; * p ≤ 0.05; ** p ≤ 0.001
Figure 1.1. Weekly food intake of SD rats during the study period of subacute exposure to thiacloprid

![Weekly food intake graph](image)

Figure 1.2. Monthly food consumption per day of thiacloprid exposed rat for 90 days

![Monthly food consumption graph](image)
Figure 1.3. Animal weight and relative liver weight after 28 and 90 days of oral administration of thiacloprid

![Graph showing animal weight and organ weight](image)

Figure 1.4. Serum enzyme parameters of rats after 28 days of thiacloprid administration

![Graph showing serum enzyme levels](image)
Figure 1.5. Serum enzyme parameters of rats after 90 days of thiacloprid administration

![Figure 1.5](image1.png)

Figure 1.6 Efect of thiacloprid on Protein and bilirubin level in serum after subchronic and subacute exposure

![Figure 1.6](image2.png)
Figure 1.7 Serum glucose concentration after 28 and 90 days of thiacloprid oral exposure

![Serum Glucose Concentration Graph]

Figure 1.8. Activity of stress marker enzymes in thiacloprid exposed rat liver after 28 days of oral intubation

![Stress Marker Enzymes Activity Graph]
Figure 1.9. Activity of stress marker enzyme in thiacloprid exposed rat liver after 90 days of oral intubation
Figure 1.10 Liver tissue of control animals showing radially arranged hepatic cords around the central vein (CV) (H&E Stain, 10X).

Figure 1.11 Liver tissue of control animals showing normal hepatocytes (H&E Stain, 100X).
Figure 1.12 Thiacloprid treated rat liver section showing vacuolar degeneration with signs of necrosis (CV – central vein) (H&E Stain, 10X).

Figure 1.13 Thiacloprid intoxicated liver tissue showing vacuolar hepatocytes degeneration (arrows indicate vacuoles in hepatocytes (H&E stain, 100X).
Figure 1.14 Histopathological appearance of thiacloprid treated liver tissue. Inset photograph with white necrotic spot on liver reflecting a necrotic patch in liver section (H&E stain, 40X).

Figure 1.15 Histopathological alterations after thiacloprid intoxication. Inset photograph showing lesion of necrotic cyst seen as gross necrosis of hepatocyte (H&E stain, 100X).
Figure 1.16 Thiaceoprid treated rat liver showing centrilobular necrosis of hepatocyte surrounding central vein (black arrow) and central vein showing cellular debris of necrotic cell (H&E stain, 40X).

Figure 1.17 Thiaceoprid treated rat liver showing infiltration of inflammatory cells (blue arrow), degenerating hepatocytes with vacuole (black arrow) and gross necrosis (H&E stain, 100X).