Chapter 4

Results and Discussion
4.0 Results and Discussion

4.1 Validation and application of the method

4.1.2 Quality control:

Each analyte was identified by matching the retention time of the sample with standard. Procedural blank, consisting of all reagents and glassware’s used during the analysis were periodically determined to check the cross contamination. The procedural blank did not show any matching or interfering peak. Validation of 48 pesticide namely OCs- α-HCH, β-HCH, α-HCH, δ –HCH, Dicofol, Aldrin, o,p-DDE, p,p-DDE, o,p-DDD, p,p-DDD, p,p-DDT, α-Endosulfan, β-Endosulfan, SPs-Fenpropathrin, λ-Cyhalothrin, Permethrin-I, Permethrin-II, β-cyfluthrin-I, β-cyfluthrin-II α-cypermethrine, Fenvalerate-I, Fenvalerate-II, Deltamethrine, OPs-Dichlorvos, Phorate, Phorate sulfone, Phorate sulfoxide, Dimethoate, Diazinon, Methyl parathion, Chlorpyrifos methyl, Fenitrothione, Malathion, Chlorpyrifos, Chlorofenvinfos, Profenofos, Ethion, Edifenphos, Anilophos, Phosalone, H-Atrazine, Atrazin, Fluchloralin, Dimethachlor, Alachlor, Pendimethalin, Butachlor and Hexaconazole was done in various food commodities.

4.1.3 Recovery

Recovery studies at three fortification level (0.05, 0.10, 0.50 mg/kg) in all food commodities under the study like vegetables, fruits, cereals, spices and milk was performed to evaluate the efficiency of extraction method. Recovery results have indicated that overall recovery of pesticide and % RSD in vegetable ranged for
OCs (70.44 - 95.65), (3.20-9.50%), OPs (70.20-95.25), (4.20-10.60%), SPs (70.25-93.40), (4.30-9.50%) H (70.22-91.11), (4.70-9.50%) in fruit ranged from OCs (70.30 to 95.55), (3.23-9.50%), OPs (70.30-93.45), (3.17-9.61%) SPs (70.80-87.00), (3.17-9.50%), H (70.50-92.35), (3.17-9.50%), in spices OCs (70.20 to 93.30), (3.17-9.50%), OPs (70.75-92.50), (3.17-9.61%) SPs (70.45-90.40), (4.50-11.50%), H (72.20-95.40), (3.35-8.90%), in cereals OCs (72.25 to 95.40), (3.23-10.50%), OPs (70.20-95.25), (3.17-9.61%) SPs (70.25-93.40), (3.17-9.50%), H (70.22-93.45), (3.17-9.50%) respectively. (Table 1 -16)

The overall recovery was more than 70 % in all food commodities for OCs, OPs, SPs and Herbicides at lower spiking level 0.05 mg/kg with RSD below 15 % which represent repeatability by QuEChERS method for all pesticides (European commission 2007)

4.1.4 Linearity

The linear relationship is calculated by un weighted linear regression, but may be fit to a weighted linear regression with weighting factors of 1/concentration (1/X) or 1/concentration$^2$ (1/X$^2$), if justified. Acceptability of the weighting factors is determined by evaluation of the pesticides across of the 5 different concentrations (0.025, 0.05, 0.1, 0.5, 1.0 mg/kg). The criterion of recommended acceptance for a standard curve is dependent upon the standard curve format. Calibration of standard curves generated by fortification of control food samples and processed through the QuEChERS procedure are subject to the same acceptance criteria as the samples. Calibration standard curves ($r^2 = 0.996 - 0.999$
for ECD and 0.997 - 0.999 for NPD) generated by standards in solvent by fortification food sample.

4.1.5 Accuracy (recovery)

The recovery experiments were carried out on food commodities by fortifying the samples 10 g in five replicates with pesticide mixture separately at three concentration levels, i.e. 0.05, 0.1 and 0.5 mg/kg. The samples were extracted with 10 ml ethyl acetate. The recoveries obtained (0.05, 0.10 and 0.5 mg/kg level), samples have indicated that overall recovery exceeded 70.20 to 95.65 % (Table 1-16).

4.1.6 Precision (repeatability)

Intraday precision was determined by injecting the GC column, with 1µl working standard solution at 3 concentration levels (0.05, 0.10 and 0.5 mg/kg) in five replicates in one day. Intraday precision was determined by measuring the same control and duplicate for 5 days. Results of these measurements showed that there was less than 10% variation in the reproducibility by this method for analysis of pesticide residues in various food commodities.

4.1.7 Limit of Detection / Limit of Quantification

The limits of detection (LODs) and quantification (LOQs) were calculated (It is the quantity of analyte that generates a response 3 times greater than the signal of noise ratio of the detection system) in accordance with Taylor (1987) and INMETRO (2007) guidelines. For this purpose, 48 independent analyses in food
sample spiked with mixture of pesticides at a level of 0.05, 0.1 and 0.5 mg/kg were performed. The limit of quantification (LOQ) is the smallest measured content of pesticides above which the determination can be made with the specified degree of accuracy and precision. The LOQ for pesticides in the present study was calculated as 0.004 to 0.033 mg/kg.

4.1.8 Level of various pesticides in food commodities

A total 250 samples of various food commodities was analyzed for the determination of forty eight multi-pesticides by QuEChERS method. The residual level and trend of occurrence of analyzed pesticide in different food commodities as follows-

4.1.9 Vegetables

Presences of pesticides in vegetable like brinjal were Dicofol (BDL - 0.019 mg/kg), Σ-endosulfan (0.174 - 0.189 mg/kg) and cypermethrin (0.261 - 0.528 mg/kg) and in cabbage been Σ-endosulfan (ND-0.257 mg/kg), malathion (0.157-3.554 mg/kg) and chlorpyriphos (2.124-0.329 mg/kg) but none of these pesticides was > MRL, except malathion. However in cauliflower Σ- HCH ( 0.573-1.593 mg/kg), malathion (ND-0.028 mg/kg), dicofol (0.856-2.310 mg/kg) and ethion (0.013-0.465 mg/kg) were detected but only in one sample dicofol was >MRL. In okra two samples contained Σ-HCH (0.0.048-0.351 mg/kg), endosulfane (ND-0.538 mg/kg), and malathion (ND-0.225 mg/kg). Tomato was found only malathion (ND-0.008 mg/kg).
4.1.10 **Fruits**

Detected pesticides in fruit like apple were cypermethrin (0.125 - 0.356 mg/kg) and in mango malathion (0.050-3.120 mg/kg) and chlorpyriphos (1.124-0.220 mg/kg) but none of these pesticides was > MRL, except malathion. However in guava malathion (BDL-0.228 mg/kg) and dicofol (0.856-3.310 mg/kg) were detected but only in one sample dicofol was >MRL. In orange two samples contained endosulfane (0.325-0.430 mg/kg), and chlorpyrephos (BDL-0.0.250 mg/kg). In papaya anilophos (ND-0.082 mg/kg), malathion (ND-0.258 mg/kg) and β-cyfluthrin (ND-0.514 mg/kg).

4.2.1 **Cereals**

Detected pesticides in wheat samples were dicofol (0.050- 0.278 mg/kg), Σ-endosulfane (0.108-0.389 mg/kg), and cypermethrin (0.361-0.630 mg/kg) their cypermethrin were >MRL. In rice samples Σ-endosulfane (0.050-0.257 mg/kg), malathion (0.250-4.504) and chlorpyriphos (2.124-0.329 mg/kg) but none of these pesticides was > MRL, except malathion. However, in pulses Σ-HCH (0.573-0.1.593 mg/kg), malathion (0.025-0.028 mg/kg) and dicofol were detected but only in one sample dicofol was >MRL.

4.2.2 **Spices**

Presences of pesticides in spices like turmeric were dicofol (BDL - 0.019 mg kg⁻¹), Σ-endosulfan (0.174 - 0.189 mg kg⁻¹) and cypermethrin (0.261 - 0.528 mg kg⁻¹) but none of these pesticides was > MRL. However in coriander, three pesticides: Σ-endosulfan (ND-0.257 mg kg⁻¹), malathion (0.157-3.354 mg kg⁻¹) and chlorpyriphos (0.329-2.124 mg kg⁻¹) were detected but only in one sample,
malathion was > MRL. In chilli four pesticides namely Σ-HCH (0.513-1.593 mg kg\(^{-1}\)), malathion (ND-0.028 mg kg\(^{-1}\)), dicofol (0.856-2.310 mg kg\(^{-1}\)) ethion (0.013-0.465 mg kg\(^{-1}\)) were detected but none of these pesticides was above > MRL except dicofol. In cumin seeds, three pesticides; Σ-HCH (0.048 - 0.351 mg kg\(^{-1}\)) Σ-endosulfan (ND-0.538 mg kg\(^{-1}\)), chlorpyriphos (ND-0.025 mg kg\(^{-1}\)) were detected but were below to MRL. In black pepper only malathion (ND-0.008 mg kg\(^{-1}\)) was detected but were below to MRL. In aniseed three pesticide namely anilophos (ND-0.042 mg kg\(^{-1}\)), malathion (ND-0.514 mg kg\(^{-1}\)) were detected while in Nutmeg only Σ-HCH (0.152-0.586 mg kg\(^{-1}\)) were detected where Anilophos and Malathion were > MRL in aniseed and Σ-HCH was below to MRL. It is interesting to note that some spices of Lucknow market basket samples like cardamom (small and big) fenugreek, canaway, dry zinger, mace, cinnamon, cuss leaf and clove have not shown the presence of any analyzed pesticide residues. However the pattern of pesticide residues present are in falling order: Chilli > coriander > cumin seed > Turmeric > nutmeg > Aniseed > Black Pepper. None of the spice samples have shown the presence of aldrin, DDT and herbicides residues. The presence of pesticide residues in spices has become a global concern. Fragmentary reports have revealed the presence of only OCs in few spices (Srivastava et. al., 2001 and Amita Rani et. al., 2003). The presence of trace level of various pesticide residues in spices may be their judicious use and proper waiting period followed by farmers. Therefore it should be mandatory for the spices industries to analyses the pesticide residues in every batch of spices so that the consumer is assured of its quality.
4.2.3 Milk

Analyzed all types of milk samples like brand-1, brand-2, brand-3 and brand-4 were collected from Lucknow local market, but none of these pesticides were detected in any samples.
4.2.4 In-life parameters

Repeated oral administration of dicofol, malathion and cypermethrin even at high doses i.e. 20, 16, 8 mg/kg/b.wt/d and their mixture did not produce any mortality during 90 days exposure. However, there was significant decrease in body weight gain of animals at highest dose (20, 16, 8 mg/kg/b.wt/d and their mixture) level. However, at high dose mixture some toxicity symptoms were observed (Table 24).

4.2.5 Food consumption

There was no change in food consumption of animals exposed with Lower doses (5, 4, 2 mg/kg/b.wt./d and its combination and Middle doses (10, 8, 4 mg/kg/b.wt./d and its combination) at 30, 60, 90 days. However, there was significant decrease in food consumption of rats exposed with high doses (20, 16, 8 mg/kg/b.wt./d and its combination) at 90 days (Table 25).

4.2.6 Neurobehavioral examination (spontaneous locomotors activity, SLA)

Lower doses of oral exposure of individual and mixture of dicofol, malathion and cypermethrin to male rats resulted in no significant alteration in various aspects of spontaneous locomotor activity at 90 days of exposure. However, a significant decrease in distance traveled, ambulatory time and stereotypic time was noted in animals exposed with i.e. mixture of middle 10+8+4 mg/kg/b.wt./d and high dose of 20+16+8 mg/kg/b.wt./d dicofol, malathion and cypermethrin respectively after 90 days. However, in contrast resting time showed a significant increase in animals. Ambulatory time significantly decreased in animals after 90 days exposure at 20+16+8 mg/kg/b.wt/d combined dose of dicofol, malathion and cypermethrin. (Table 26).

4.2.7 Hematological studies

There were no significant changes in hematological parameters of male rats orally administered lower, middle, higher and its combination doses for 90 days.
No changes were observed in RBC, WBC, Hb, MCV, HCT and differential leukocyte counts of the treated animals as compared to controls (Table 27).

4.2.8 Relative organ weights

The relative organ weights of vital organs of males are shown in (Table 28). The vital organs like brain, liver, heart, lung, kidney, spleen, testis, adrenal, epididymis and seminal vesicle of rats did not illustrate any change in their relative organ weights at any doses as compared to controls. However, relative weight of liver, kidney and brain was significantly increased (p< 0.05) at 20+16+8 mg/kg/b.wt/d dose.

4.2.9 Biochemical studies

The results of serum biochemical parameters of male rats orally administered dicofol, malathion and cypermethrin for 90 days are shown in Table 29. Serum biochemical parameters such as GOT, GPT, glucose, BUN, total protein, albumin, globulin, bilirubin, cholesterol and creatinine were not significantly changed in animals exposed to 5, 4, 2 and its combination mg/kg/b.wt./d and 10, 8, 4 and its combination mg/kg/b.wt./d doses as compared to controls. However, a significant increase (p < 0.05) was noted in serum GOT, GPT, glucose and blood urea nitrogen in animals exposed to 20+16+8 mg/kg/b.wt./d combined dose.

4.2.10 Histopathology

Combined exposure of high dose of dicofol, malathion and cypermethrin (20+16+8 mg/kg/b.wt./d) for 90 days produced necrosed purkinji cells with loss of dendrites and granules in granular layer of cerebellum in male rats as compared to controls (Fig. 1A and 1B). The hepatocytes of liver showed mild focal necrosis with swollen cellular nuclei and cytoplasmic lesions at combined exposure of high dose (Fig. 2A and 2B). There were slight degeneration of tubules and glomeruli of kidney of the male rats at higher doses as compared with controls (Fig. 3A and 3B). However, no pathological changes were observed in brain, liver and kidney of rats exposed to lower and middle individual and combined doses of dicofol,
malathion, cypermethrin at 5, 4, 2, mg/kg/b.wt./d and 10, 8, 4, mg/kg/b.wt./d doses.

4.3.1 Effect of dicofol, malathion, cypermethrin and its combination on LPO

Level of LPO measured in term of MDA was not altered in liver brain and kidney of male rat orally administered at 5, 4, 2 individual and combination mg/kg/b.wt./d of dicofol, malathion, cypermethrin. A significant increase in level MDA was found in liver and kidney at middle and higher combination doses of 10+8+4 mg/kg/b.wt./d & 20+16+8 mg/kg/b.wt./d of dicofol, malathion and cypermethrin, as compared to control. Level of increase was 30% in liver and 25% kidney and 10% in brain (Figure 4.1 – Figure 4.3).

4.3.2 Effect of dicofol, malathion, cypermethrin and its combination on GSH

Significant decreases in GSH content was observed in liver of rat exposed to higher combination 20+16+8 mg/kg/b.wt/d dose of dicofol, malathion and cypermethrin. However there was no change in GSH content in liver, brain and kidney at lower, middle individual and combined dose levels (Figure 4.1 – Figure 4.3).

4.3.3 Effect of dicofol, malathion, cypermethrin and its combination on SOD activity

Significant decreases in SOD content was observed in liver of rat exposed to higher combination 20+16+8 mg/kg/b.wt/d doses of dicofol, malathion and cypermethrin. However there was no change in SOD in liver, brain and kidney at lower, middle individual and combined dose levels. Present level of decreases in SOD activity was 50% in liver and 30% in brain at higher dose of combination 20+16+8 mg/kg/b.wt/d dose (Figure 4.1 – Figure 4.3).

4.3.4 Effect of dicofol, malathion, cypermethrin and its combination on CAT activity
A significant decrease in activity of CAT was observed in liver and brain of male rat exposed to combination of 20+16+8 mg/kg/b.wt./d. The level of decrease in CAT activities was 40% in liver and 25% in brain respectively. However no significant changes in CAT activity were found in following tissues at 10+8+4 mg/kg/b.wt./d doses level (Figure 4.1 – Figure 4.3).

4.3.5 Effect of dicofol, malathion, cypermethrin and its combination on GPx activity

A significant decrease in activity of GPx was observed in liver and brain of male rat exposed to combination of 20+16+8 mg/kg/b.wt./d. The level of decrease in GPx activities was 14% in liver and 10% in brain respectively. However no significant changes in GPx activity were found in tissues at lower and middle doses (Figure 4.1 – Figure 4.3).

4.3.6 Induction of chromosomal aberration by dicofol, malathion, cypermethrin and its combination

The percent incidence of aberration in positive control i.e. B(a)P treated cells was recorded to be 25.84% at 50 mg/kg/b.wt./d dose as compared to 1.78% in untreated cells (Figure 4.4 and Figure 4.5). The frequency of aberration in dicofol, malathion, cypermethrin and its combination exposed animal cells was 5.68%, 7.68%, 10.03% and 12.84% respectively at higher doses of above pesticides respectively, indicating the significant (p<0.05) genotoxic potential for BM.

4.3.6 Induction of micronucleus formation by dicofol, malathion, cypermethrin and its combination

A significant (<0.05) dose dependent increase in number of micronuclei was recorded in BM following dicofol, malathion, cypermethrin individual and its combination exposure. The frequency of micronuclei (MN/1000 cells) was raised up to ~41 at 5 μg/ml of positive control i.e. B(a)P exposure, whereas in untreated control the frequency was 1.78. Dicofol, malathion, cypermethrin and its
combination exposure (20, 16, 8 and 20+16+8 mg/kg/b.wt./d) led to induction of micronuclei frequency in BM up to 6, 8, 10 and 13%, respectively (Figure 4.7), confirming the genotoxic potential of dicofol, malathion, cypermethrin individual and its combination. The flow cytometry studies also confirmed the induction of micronuclei by dicofol, malathion, cypermethrin individual and its combination exposure (Figure 4.6). A significant (p<0.05) increase in frequency of micronuclei was also observed in BM with combination exposure of dicofol, malathion and cypermethrin by conventional method. The percent of micronuclei for control and for the 20, 16, 8 and 20+16+8 mg/kg/b.wt./d dicofol, malathion, cypermethrin and its combination exposure were 1.46, 3.40, 4.00, 4.5 respectively, thus complementing the results obtained earlier.

4.3.7 Determination of molecular target of pesticides using In-silico tools

The top 8% TarFisDock output identified 90 protein targets with binding affinities to these pesticide over -30.04 kcal mol\(^{-1}\). After testing each one with AutoDock Vina program, top 2% of the target list were selected according to the docking score (Table 30), and those with a binding affinity over -7.0 kcal mol\(^{-1}\) and CDocker score 38.09 kcal mol\(^{-1}\) were chosen for the analyses. RMSDs values calculated for the ligand position in the protein as given by TarFisDock, AutoDock and CDocker.

Based on the docking score, we found that pesticide (dicofol, malathion and cypermethrin) could interact in an aryl hydrocarbon receptor (AhR)-independent way with different enzymes such as hydrolases, isomerases, oxidoreductases, oxidases and other receptors. As can be seen from (Table 1), proteins with the greater binding affinity were neutrophil collagenase (MMP8) stromelysin-1 MMP3, oxidosqualene cyclase, and myeloperoxidase (Figure 5.4). For these proteins, the binding sites for pesticide are embedded in a hydrophobic region, with interactions of both the chlorine atoms and the aromatics rings.

AutoDock Vina - generated docking of pesticide with an available theoretical AhR model. The binding site is also hydrophobic with prevalence of aromatic and
aliphatic residues. However, the calculated affinity (−7.5 kcal mol⁻¹) is not as good as the ones observed for other these pesticide targets predicted by TarFisDock.

4.3.8 Discussion on analytical results

Pesticide exposure is a global public health issue as widespread use and release into the environment poses deleterious effects on animal and human health because of their toxicity (Weiss et. al., 2004; Calvert et. al., 2008). According to the World Health Organization, 3 million cases of pesticide poisoning occur every year, resulting in more than 250,000 deaths (Yang and Deng, 2007). Despite this alarming figure, there is currently no global system to track and stop poisoning or diseases associated with pesticide use (Ali and Chia, 2008). In present study an attempt has been made to determine the residual level of forty eight pesticides in vegetable, fruits, cereals, spices and milk samples to assess the pesticide intake due to consumption of these commodities.

A total 250 samples of various food commodities were pesticide analyzed. The pesticides residues were found in 35.2 % of analyzed samples. The trend of detected pesticides in food commodities had shown in (Figure 4.1). The presence of pesticides residues in animal feeds is the main source of pesticide contamination of dairy products. Other factors may include environmental contamination, application of pesticides on farm animals for ectoparasite removal and accidental spills. The best way of controlling the contamination of milk from pesticides residues is to prevent contamination of feed. Based on the limited data on animal exposure via feed produced according to good agricultural practice, it is not likely that ruminant animals will be exposed to levels that could cause toxic effects.

The vegetables, fruits, cereals, spices and milk under study are used for dietary intake. Pesticides are the chemicals applied on them. The present investigation determined the pesticide residues in various food commodities collected from markets basket samples of Lucknow city, India and compared with the maximum residues limits (MRL) set by the Prevention of Food Adulteration Act (PFA) 1954. Table 1-16 shows the MRL of these analysed pesticides in vegetables, fruits, cereals, spices and milk. Residues occurred in 48% of vegetables, 34% of
fruits, 34% of cereals, 37.5% of spices and none of milk respectively. Residues of OCs, OPs and SPs along with fungicide and herbicides in various food commodities like; vegetables, fruits, cereals, spices and milk in India and abroad were determined frequently to know the status and possible health hazard (Frank et al. 1987, Nath et al. 1990; Dikshith et al. 1992; Srivastava et al. 2001; Kumari et al. 2002, 2003a; Amita Rani et al. 2003; Shahi et al. 2005; Darko et al. 2008, Srivastava et al. 2011). Various studies have been shown that residual level above MRL (PFA, 1954) show hazard index above on the consumption basis. (Arora et al. 2009; Chen et al. 2011, Osman et al. 2011; Upasana et al, 2013). In the present study most frequently detected pesticide are dicofol, malathion and cypermethrin and showed the residual level above MRL (PFA, 1954) (Table 19 - 22). In vegetables like; brinjal, endosulfan (0.680 mg/kg) and cypermethrin (0.528 mg/kg), cabbage; malathion (3.554 mg/kg), in cauliflower; dicofol (2.310 mg/kg) were detected above MRL (PFA, 1954) Table 19. In fruits OPs were frequently detected above MRL, in mango guava and papaya malathion detected above MRL in range of (0.228-3.120 mg/kg) Table 20. In cereals cypermethrin was detected above MRL (0.630 mg/kg) in wheat, in rice malathion was above MRL (4.504 mg/kg) and in pulses dicofol was above MRL (3.010 mg/kg), malathion was also in higher level but its MRL value was not available in PFA, 1954 list (Table 21). In spices like coriander; malathion was above MRL (3.554 mg/kg), chilli; dicofol (2.310 mg/kg) and in aniseed malathion (0.258 mg/kg) and anilophos (0.042 mg/kg) were found above MRL (Table 22). However in recent times, predominance of cypermethrin residues in fruits and vegetables was also reported by several workers (Singh and Kiran Singh 2004; Chetan et al. 2011; Lozovicka et al. 2012) in their respective studies area indicating their excessive use.

The result of present investigation further support the findings of the studies carried out in India and abroad. Residues of cypermethrin in fruits and vegetables were also reported (Colume et al. 2001; Kumar et al. 2006). A another study was also carried out in Danish Market which indicated residues of pyrethroids insecticides in 54% samples of fruits and 30% vegetables (Andersen and paulsen, 2001). A simple and rapid chromatography method also proved the occurrence of

In our study dicofol, malathion and cypermethrin were found above from their respective MRL in most of the samples. Real world exposure to pesticides normally occurs through lower level single or repeated exposure for example, as residues in food products (Zheng et al., 2000). Hence, 90 days oral residual toxicity studies on mixture of these three pesticides namely dicofol, malathion and cypermethrin was conducted in rat model and lowest exposure dose was selected with reference to their respective MRLs to find out the synergistic effects of mixture of these chemical pesticides at lowest doses.

4.3.9 Discussion on toxicity results

Toxicity of OCs, OPs, SPs and Hs has been reported time to time by various researches (Prasad et al. 2008, El-Kashoury et al. 2010, Nahid et al. 2009, Abdel-Tawab et al. 2012 ). OCs has been found to cause effects on immunological, reproductive, respiratory, hematological and genetic systems. OPs and SPs including malathion and cypermethrin are known to induce oxidative tissue damage (Jou et al. 2004, 2007 Kannan et al. 2003).

Our result, shows that 90 days oral exposure of high dose combination of dicofol, malathion and cypermethrin to male rats has produced significant toxic effects, the toxicity of combination of these has not been reported. It is interesting to know that there was significant decrease in the body weight of high dose exposed rats together with reduced food consumption and increased liver weight. The weight gain in animals serves as index of growth rate (Palani et al., 1999). The reduced food consumption and increased liver weight of high dose exposed animals seems to be due to toxic potential of combination of dicofol, malathion and cypermethrin. The significant increase in weight of liver was, however, found to be associated with concomitant increase in the activity of GOT and GPT in serum (Table 29). It is important to note that the elevated activity of serum GOT and GPT recorded in the study may be due to liver damage. This has been confirmed
by hepatocellular damage in the combination of higher dose treated animals. Our present knowledge on liver changes induced by combination of dicofol, malathion and cypermethrin is both limited and equivocal (Eiben and Rinke, 1989). In contrast from the previous finding of others (Eiben, 1988; Bomann, 1991; Pauluhn, 2003), the food consumption is significantly decreased at higher dose levels. The reduced effects on the body weight of the high dose treated rats may not be clearly treatment related and could be attributed to food palatability problems in animals. However, increased level of blood glucose may also be the indication of mild combination of dicofol, malathion and cypermethrin induced changes in carbohydrate metabolism. No significant changes in cholesterol indicated that combination doses has not attributed in fat metabolism. Interestingly the elevated levels of blood urea nitrogen together with tubular changes in the kidney and its increased weight at higher dose exposed rats have also indicated its nephrotoxic effects. Haschek and Rousseaux (1998) have also reported cell necrosis and cell infiltrate in the form of cytoplasmic changes, swelling of hepatocyte nuclei are often caused by increased liver manifestations.

Significant decrease in spontaneous locomotor activity in the rats treated with the high dose of combination of dicofol, malathion and cypermethrin has indicated the accumulation of cypermethrin in the brain. Chao and Casida (1997) has reported the accumulation of cypermethrin in mouse brain following direct intra peritoneal administration. However, Brunet et al. (2004) have also reported that malathion and cypermethrin is highly absorbed in human intestinal cell suggesting its potential effects.

Abou-donia et al., 2008 showed significant sensory motor impairments (beam walk time, inclined plane performance, and forepaw grip) after maternal exposure of sublethal dose of imidacloprid during gestation of rats. These neurobehavioral deficits may reflect dysfunction at multiple anatomical areas in central nervous system. The pathological changes in brain seen in the present study suggest that multiple brain region abnormalities may be involved in changes of spontaneous locomotor activity. Earlier studies have shown that concentration of pesticides in
brain generally correlate with the severity of toxicity and symptoms of neurotoxicity which were found to increase with the pesticide concentration in brain (Sheets, 1994; Nagata et al., 1996). Necrosed purkinje cells and loss of granules in the granular layer of cerebellum have also provided support to the neurobehavioral effects indicating accumulation of malathion and cypermethrin in the brain.

The results of the genotoxicity revealed that combination of dicofol, malathion and cypermethrin causes significant induction of CAs and MN in a higher (20+16+8 mg/kg/b.wt./d) has been reported to have genotoxic effects. In this study, we report for the first time the genotoxic potential of dicofol, malathion and cypermethrin in wistar rat (Tables 1 and 2). The frequency of CAs is a sensitive cytogenetic assay for detecting exposure to mutagens and carcinogens (Lodovici et al., 1996; Bonassi et al., 1995; Hour et al., 1998). The dicofol, malathion and cypermethrin induced CAs recorded in the present study may be due to early changes that either caused an increase in induced DNA damage or interfered with their repair mechanism. Micronuclei induction is considered to be another sensitive biological indicator of genotoxicity (Heddle et al., 1991). The formation of MN is related to the loss of acentric chromatin fragments and/or whole chromosomes (Fenech, 2000). Our results suggest that the increase in the frequency of MN in the combination of higher dose dicofol, malathion, cypermethrin combination and cypermethrin individual (8mg/kg/b.wt) exposed animals may be the consequences of either direct DNA damage or mitotic spindle break down (Fenech, 2000; Lohani et al., 2002, 2003). However, another possibility suggests that combination of dicofol, malathion and cypermethrin exposure may also cause an aneugenic mode of action because it inhibits cell division and the mitotic spindle apparatus (Fenech, 2008).

A large number of xenobiotic have a capability to generate free radicals in biological systems raising question whether oxidative stress is major concern for tissues damage. However antioxidant enzymes like SOD, CAT and GPx have effect on oxidant molecule on tissues and are active in defense against oxidative
cell injury by means of there being free radical scavengers. Pesticide mediated toxicity involve excessive production of ROS leading to alteration in the cellular antioxidant defense system and consequently affecting susceptibility to oxidative stress (Lopez et.al., 2007). Pesticide also induces free radical generation that lead to DNA damage, protein degradation, LPO and finally culminating into damage to various vital tissues like liver, brain and kidney (Banerjee et.al 1999, Khan et.al 2005). These elevated free radicals and depressed antioxidant defense may lead to disruption, oxidative damage to cell membrane and hence increase susceptibility to LPO (Kapoor et.al., 2010). Mixed exposure to limited data on induction of free radical scavenging enzyme in biological tissues following mixture of dicofol, malathion, cypermethrin administration, hazard arising from its exposure are of great interest. Increase MDA suggests an increase production of free O$_2$ radicals in rat (Mansur and Mossa 2009). In present study combination of doses significantly induce LPO and decreases other vital antioxidant system in liver, brain and kidney at high dose. Susceptibility of liver, brain and kidney to this stress due to exposure of pesticide is function of overall balance between degree of oxidative stress and antioxidant capacity (Khan et.al., 2005). High level of LPO in liver in the present study suggested the production of oxidative metabolites or free radical during hepatic metabolism may be due to progressive nature of free radical chain reaction.

O$_2$ free radical and hydroperoxide collectively termed as ROS are produced by univalent reduction of dioxygen to superoxide anion (O$_2^-$) which intern converted in to H$_2$O$_2$ and O$_2$ through a reaction catalyzed by SOD (Rai and Sharma 2007). Antioxidant enzyme SOD and catalase constitute first line of defense against deleterious effect of oxy radicals in cell by catalyzing dismutation of superoxide radical. Decrease in activity of SOD in liver, brain and kidney of pesticide mixture exposed rats may be due to consumption of this enzyme to scavenge O$_2$ free radical to H$_2$O$_2$. Similar decrease activity of SOD in animal was also reported with the exposure of cypermethrin, malathion and chlorpyriphos (Khan et.al 2005; Rai and Sharma et al., 2007; Mansour and Mossa 2009)
Various studies have demonstrated decrease in GPx activity due to xenobiotics and supported our finding (Mansour and Mossa 2009; Kapooe et al., 2010). GSH play a key role in modulating pesticide induced oxidative damage in tissues. GSH depletion is evident to intensify LPO and pre dispose cell to oxidant damage (Khan et al., 2005). A significant depletion of GSH in liver together with decrease in activity of GSH in liver, brain and kidney at middle and high combination (10+8+4 mg/kg/b.wt/d), (20+16+8 mg/kg/b.wt/d) dose of dicofol, malathion and cypermethrin may induce oxidant damage in tissue of rat. In addition GSH participate in detoxification of xenobiotics as substrate for enzyme glutathion-S- transferase (GST), which is reduced by middle and high combination 10+8+4 mg/kg/b.wt/d, 20+16+8 mg/kg/b.wt/d dose of dicofol, malathion and cypermethrin may be through direct utilization of GSH as an antioxidant in terminating free radical reaction (Sharma et al., 2005; Rehman et al., 2006; Rashid et al., 2006).

The decreased activity of SOD, GPx, CAT and GSH content together with increased LPO may be attributed to the induced free radicals in middle and high combined dose of dicofol, malathion and cypermethrin treated rats. The toxicity of many xenobiotics is associated with the production of free radicals which are not only toxic themselves, but may also implicate in the pathophysiology of many diseases (Abdollahi et al., 2004)

4.3.10 Discussion on toxicity results by data base docking of virtual screening

The virtual screening of protein targets for pesticide has shown that several of them have the potential to bind this toxic compound. Although the physiological and toxicological relevance of this finding is still unknown, it brings the opportunity to rethink new mechanisms by which pesticide could be interfering with many biochemical systems in the body.

The highest binding score for pesticide binding was obtained for metalloproteinase 8 (MMP8), known as neutrophil collagenase. It is a member of the interstitial collagenase family, capable of degrading fibrillar type I, II, and III collagens. Its expression and activity has been associated with chronic
inflammatory and fibrotic diseases such as cystic fibrosis, rheumatoid arthritis, periodontal disease, and chronic skin wounds (Matsuki et al., 1996; Nwomeh et al., 1999; Allport et al., 2002; Ratjen et al., 2002). The MMP3 of ectopic endometrium may participate in the process of invasion and tissue remodelling that is hypothesized to occur in the pathogenesis of endometriosis (Cox et al., 2001). Pesticide also affects the pathophysiology of endometriosis by modulation of immune and endocrine function (Pauwels et al., 2001).

Oxidosqualene cyclase catalyzes the cyclization of squalene 2,3-epoxide to form the tetracyclic lanosterol, the precursor to all sterols (Kramer, 2006).

Myeloperoxidase (MPO) is a lysosomal enzyme that is found in white blood cells and neutrophils, and its primary function is to mediate a defense response against invading pathogens (Shetty et al., 2006). MPO uses hydrogen peroxide and chloride to produce hypochlorous acid (HOCl), a potent oxidant that then reacts with proteins, DNA, and lipids to cause cellular injury.

Cytosolic beta-glucosidase (hCBG), is present in the liver, kidney, intestine and spleen of humans but primarily in enterocytes (Daniels et al., 1981). This enzyme hydrolyses many common dietary xenobiotics, including glycosides of phytoestrogens, flavonoids, simple phenolics and cyanogens (Berrin et al., 2002). Finally, acetylcholinesterase (AChE) is the enzyme involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firings. It is vital for normal functioning of sensory and neuromuscular systems (Murphy, 1986). Although it is a specific molecular target for organophosphate and synthetic pyrethroids pesticides, its binding capacity to portions is surprising.

In short, although it is safe to state that this virtual screening approach provides insight about hypothetical targets for pesticide, it is also worth emphasizing that currently, the use of these tools might have limitations, especially when considering environmental samples where activity depends on mixtures of pesticide-like compounds.

The all result of present study have indicated that lower dose of dicofol, malathion and cypermethrin individual and combination has not induced oxidative
stress at 5, 4, 2 mg/kg.b.wt./d and their combination 5+4+2 mg/kg.b.wt./d and middle dose of its individual and high dose of individual doses to male rat when exposed for period for 90 days. However, 10+8+4 mg/kg/b.wt/d and 20+16+8 mg/kg/b.wt/d combined dose of dicofol, malathion and cypermethrin has significantly induced oxidative stress to male rat.

It is evident by earlier workers that lowest observable effect level (LOEL) of dicofol, malathion and cypermethrin based on insignificant changes in reduction of body weight and other related parameters. In view of parameters such as development of signs of intoxication and mortality, organ body weight ratio, hematology, pathomorphology, enzymatic changes, chromosomal aberration and neurobehavioral examination of experimental rats it may be suggested that dicofol, malathion and cypermethrin was individual 5, 4, 2 mg/kg/b.wt./day with combination 5+4+2 mg/kg/b.wt/day produced no desinerable effects, therefore this dose may be considered as no observed effect level (NOAEL) and 10, 8, 4mg/kg/b.wt/day with combination of 10+8+4 mg/kg/day dose may be considered as lowest observable effect level (LOAEL) to male rats.