6.0 SUMMARY AND CONCLUSION

All people are inevitably exposed to pesticides, through environmental contamination or occupational use. Occupational exposure occurring at all stages of pesticide formulation, manufacture and application involves exposure to complex mixtures of different types of chemicals, active ingredients and by-products present in technical formulations such as impurities, solvents and other compounds produced during the storage procedure. Pesticides constitute a heterogeneous category of chemicals specifically designed for the control of pests, weeds or plant diseases. Although there are benefits of pesticides, drawbacks are also there, such as potential toxicity to humans and other animals. Prominent pesticide families include organochlorines, organophosphates, and carbamates. Biochemical, DNA and Electron Microscopic changes were determined in carbamate exposed workers.

Genotoxic potential of pesticides is a primary risk factor for long-term effects such as carcinogenic and reproductive toxicology. The majority of pesticides have been tested in a wide variety of mutagenicity assay covering gene mutation, chromosomal alteration and DNA damage. Pesticides have been considered potential chemical mutagens: experimental data revealed that various agrochemical ingredients possess mutagenic/genotoxic properties. Malathion and parathion are pesticides that are widely used in agriculture and are themselves of low toxicity. However, their absorption or ingestion into the human body readily results in their metabolism to malaoxon and paraoxon respectively, which are more toxic than their parent compounds and are known to cause oxidative stress which ultimately leads to DNA damage.

Nutraceuticals are natural bioactive, chemical compounds that have health promoting, disease preventing or medicinal properties. They may range from isolated nutrients, herbal products, dietary supplements and diets to genetically engineered foods and processed products such as cereals, soups and beverages. Curcumin (diferuloylmethane), a polyphenol is biologically active ingredient of *Curcuma longa* and has received attention mostly due to its antioxidant, anti-inflammatory, anti-tumoral, apoptosis-inducing, and anti-angiogenesis effects and antigenotoxic effects, which are
reported in many investigations. It has been shown to inhibit lipid peroxidation using linoleate, a polyunsaturated fatty acid that is able to be oxidized and form a fatty acid radical. In addition to inhibiting lipid peroxidation, curcumin demonstrates free radical-scavenging activity. Curcumin is also reported to reduce the DNA damage induced by tinidazole (A drug used for treatment of trichomoniasis, giardiasis and amebiasis) and gamma-radiation in human lymphocytes culture under in vitro conditions which suggest the antigenotoxic properties of curcumin.

Carvacrol is a monoterpenoid phenol and is present in the essential oils of Origanum vulgare, thyme, pepperwort, and wild bergamot. As well known, it has been shown to exhibit antimicrobial, antimutagenic, antiinflammatory, antioxidant, antitumor and antigenotoxic activities. Antimutagenic effect of carvacrol may be due to its antioxidant nature. Carvacrol has been shown to exhibit protective effect against oxidative stress and DNA damage caused by U.V. radiations in cultured lymphocytes. Sister chromatid exchange induced by Mitomycin are greatly reduced by carvacrol supplementation to cultured lymphocytes suggested its antigenotoxic effect.

Biomarkers are any substances, structures or processes that are measured to indicate an exposure or susceptibility or that predict the incidence or outcome of disease. The measurement of biomarkers, in combination with other data, plays an integral role in identifying exposure and potential human health effects. Biomarkers are thought to reflect disease risk in individuals because they indicate exposure of causative agents or represent early stage in development of disease. There are three types of biomarkers are used for assessment of genotoxicity. Biomarkers of exposure are the toxic agents or their metabolites that can be measured in body or after excretion from body. They can be measured from patient’s blood or urine. Biomarkers of effects are the effects of genotoxic agents that can be detected by various assays. These effects leads to change individual’s genetic material hence can be detected by measuring change in genetic material. Different biomarkers of effect such as sister chromatid exchange (SCE), alkaline single-cell gel electrophoresis or comet assay, chromosomal aberrations (CA), micronucleus (MN) can be used to detect the genotoxic effect of pesticides.
Different biomarkers of effect such as SCE, alkaline single-cell gel electrophoresis or comet assay can be used to detect the genotoxic effects of pesticides. SCE are interchanges of DNA replication products between sister chromatids at apparently homologous loci, suggested to represent homologous recombination repair of DNA double strand breaks. Single cell gel electrophoresis (SCGE) technique, also known as comet assay, is a sensitive, simple and rapid technique for detecting DNA single and double strand breaks, alkali labile sites, incomplete excision repair sites, and genomic structural discontinuities. While biomonitoring studies employing cytogenetic techniques are limited to circulating lymphocytes and involve proliferating cell populations, the comet assay can be applied to proliferating and non-proliferating cells. This method, within a short time, has found wide usage in epidemiological and biomonitoring studies in humans, to determine DNA damage, as a result of endogenous factors and lifestyle, occupational and environmental exposures.

Biomarkers of susceptibility are indicators of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific chemical substance. These biomarkers provide an indication of the extent to which an individual may be prone to progress from exposure to developing an adverse health effect. It is increasingly clear that genetic differences among individuals may play a primary role in susceptibility to environmentally or occupationally induced diseases and these differences are mainly due to polymorphic nature of xenobiotic-metabolizing enzymes (XME). The metabolism of pesticides is mediated at the initial oxidative step by CYP family gene products such as CYP1A1, CYP2E1. After that step, these are detoxified by conjugation to glutathione. This step is catalysed by glutathione-S-transferases (GST) enzymes which are the protein products of GST genes such as GSTM1, GSTT1. Individual variations in polymorphic genes involved in xenobiotic metabolism and DNA repair are linked to increased risk of cancer in several case-control studies. These individual differences may be important in the estimation of the risk to humans caused by the exposure to environmental toxicants like pesticides.

**Genotoxicity of pesticides (Malathion and Parathion)**

In our study, we investigated the genotoxicity of organophosphate pesticides (malathion and parathion) in cultured human peripheral blood lymphocytes (PBL) using
SCE and comet assay as a biomarker of DNA damage under in vitro conditions. To achieve this goal, blood samples were taken from 70 healthy individuals with their consent. Subjects having exposure to diagnostic X-rays, drug intake and vaccination in last 6 month prior to blood sampling have not been included in study. All the participants were asked to sign an informed consent for their participation and a questionnaire got filled by the donors for their health status. The questionnaire included the information about sex, age, diet, consumption habits, health status etc. of the blood donors. The research protocol was approved by human ethical committee of Kurukshetra University, Kurukshetra, Haryana, India.

Cultured PBL had shown the dose dependent increase in SCE frequency and Tail moment (TM) as the concentration of malathion had been increased from 10-30µg/ml. Maximum significant (P<0.05) damage to PBL was observed at 30µg/ml of malathion with maximum mean value of SCE and TM i.e. 6.55±0.54 and 7.08µM±0.16 respectively. However, at higher concentrations of malathion, PBL were failed to grow.

Similarly, parathion was also found to be genotoxic to cultured PBL and had shown DNA damage in dose dependent manner. As the concentration of parathion was increased from 0.5-2.5µg/ml, frequency of SCE and TM had also been increased and reached maximum at 2.5µg/ml (SCE-10.86±0.40 and TM-8.78µM ±0.19) indicating the maximum significant (P<0.05) DNA damage. While on further increasing concentration of parathion, PBL did not grow.

Antigenotoxicity of curcumin and carvacrol against pesticides (malathion and parathion) induced genotoxicity using SCE frequency

We studied the antigenotoxic effect of curcumin and carvacrol by analyzing the reduction in SCE frequency in presence of malathion and parathion under in vitro conditions. Malathion induced the maximum DNA damage at 30µg/ml while parathion showed the maximum damage to lymphocytes at 2.5µg/ml. Both of these maximum damaging concentrations of malathion and parathion (30 and 2.5µg/ml respectively) were used to investigate the antigenotoxic effect of curcumin and carvacrol in cultured PBL.
Antigenotoxic effect of curcumin against genotoxicity induced by malathion was analyzed by reduction in SCE frequency. PBL treated with 30µg/ml of malathion had shown the mean value of SCE 7.67±0.37 which is significantly (P<0.05) higher than the mean SCE (2.67±0.13) value of untreated sample. However, curcumin at the concentration of 25 and 50 µg/ml along with 30µg/ml of malathion had significantly (P<0.05) reduced the mean value of SCE to 5.02±0.17 and 3.53±0.23 respectively as compared to malathion treated lymphocytes.

Curcumin had also shown protective effect against the parathion induced DNA damage in cultured PBL. Parathion at 2.5 µg/ml had significantly (P<0.05) increased the mean value of SCE (5.37±0.36) as compared to mean SCE value of untreated sample (2.33±0.16). When PBL were treated with 10 and 15µg/ml of curcumin along with 2.5µg/ml of parathion, the mean value of SCE was reduced to 4.59±0.38 and 3.28±0.24 respectively and this reduction in SCE frequency was statistically significant (P<0.05) as compared to parathion treated lymphocytes.

Carvacrol was found to be antigenotoxic against genotoxicity induced by malathion to PBL. As compared to untreated lymphocytes having mean value of SCE (2.72±0.22), the lymphocytes treated with 30µg/ml of malathion had significant (P<0.05) higher mean value of SCE (7.06±0.38). Although when the PBL were treated with 2.5 and 5.0µg/ml of carvacrol along with 30µg/ml of malathion, the mean values of SCE were reduced to 5.27±0.16 and 3.65±0.17 respectively. This reduction in SCE frequency with carvacrol was statistically significant (P<0.05) as compared to malathion treated lymphocytes.

Similarly, carvacrol had also shown the antigenotoxic effect against genotoxicity induced by parathion in cultured PBL. Untreated lymphocytes had the mean value of SCE 2.46±0.16 while parathion at 2.5µg/ml had significantly (P<0.05) increased the mean value of SCE to 5.63±0.52 supporting its genotoxic nature. Carvacrol at 2.5 and 5.0µg/ml along with 2.5µg/ml of parathion had significantly (P<0.05) reduced the mean value of SCE to 4.33±0.12 and 3.07±0.14 respectively. This reduction in SCE frequency supported the antigenotoxic nature of carvacrol.
We also studied the combinatorial antigenotoxic effect of curcumin and carvacrol against both the pesticides (malathion and parathion). Treatment of PBL separately with 25µg/ml of curcumin and with 2.5µg/ml of carvacrol in presence of 30µg/ml of malathion had resulted in mean values of SCE frequencies 5.02±0.17 and 5.27±0.16 respectively whereas combination of 25µg/ml of curcumin and 2.5µg/ml of carvacrol against 30µg/ml of malathion had reduced the mean value of SCE frequency to 4.62±0.28. Likewise, PBL treated separately with 50µg/ml of curcumin and with 5.0µg/ml of carvacrol in presence of 30µg/ml of malathion had shown mean values of SCE frequencies 3.53±0.23 and 3.65±0.17 respectively. Combination comprising of 50µg/ml of curcumin and 5.0µg/ml of carvacrol against 30µg/ml of malathion had further reduced the mean value of SCE frequency to 3.26±0.20. However, this reduction in SCE frequency by the combinations of curcumin and carvacrol was found to be statistically non significant (P>0.05) as compared to their separate treatments.

Combinatorial antigenotoxic effect of curcumin and carvacrol was also analyzed against parathion. PBL treated separately with 10µg/ml of curcumin and with 2.5µg/ml of carvacrol in presence of 2.5µg/ml of parathion had shown mean values of SCE frequencies 4.59±0.38 and 4.33±0.12 respectively whereas mean value of SCE frequency was increased to 5.08±0.41 by the combination of 10µg/ml of curcumin and 2.5µg/ml of carvacrol. Similarly, treatment of PBL separately with 15µg/ml of curcumin and with 5.0µg/ml of carvacrol in presence of 2.5µg/ml of parathion had resulted in mean values of SCE frequencies 3.28±0.24 and 3.07±0.14 respectively. However, the combination of 15µg/ml of curcumin and 5.0µg/ml of carvacrol against 2.5µg/ml of parathion enhanced the mean value of SCE frequency to 3.68±0.31. We observed a small decrease or increase in SCE frequency with combination of curcumin and carvacrol as compare to their separate treatment against malathion and parathion. However, this small increase or decrease was found to be non significant.

Antigenotoxic potential of curcumin and carvacrol against genotoxicity induced by pesticides (malathion and parathion) using comet assay

Antigenotoxic effect of curcumin and carvacrol were analyzed by measuring extent of DNA damage quantitatively as tail moment (TM) value in presence of malathion and parathion. Both of the maximum damaging concentrations of malathion
and parathion (30 and 2.5µg/ml respectively) were used to investigate the antigenotoxic effect of curcumin and carvacrol in cultured PBL using comet assay.

Antigenotoxic effect of curcumin against malathion was analyzed by reduction in TM value in presence of malathion and parathion. PBL treated with 25 and 50µg/ml of curcumin along with 30µg/ml of malathion had mean TM value 4.72µM±1.34 and 0.99µM ±0.22 respectively which are significantly (P<0.05) lower than the mean TM value (8.08µM ±2.27) of malathion treated lymphocytes.

Curcumin was also found to be antigenotoxic against DNA damage induced by parathion. Parathion induced the DNA damage as shown by the increased mean TM value (8.15µM ±3.02) in comparison to mean TM value (1.53µM ±0.91) of untreated sample. While PBL treated with 10 and 15µg/ml of curcumin had shown significantly reduced mean TM values 5.23µM ±2.29 and 2.65µM ±1.70 respectively.

We also studied the antigenotoxic effect of carvacrol against both the pesticides (malathion and parathion). Like curcumin, carvacrol at 2.5 and 5.0µg/ml along with malathion had significantly (P<0.05) reduced the DNA damage (Mean TM values are 6.38µM ±1.55 and 1.02µM ±0.64 respectively) in comparison to PBL treated with 30µg/ml of malathion only (Mean TM value is 10.47µM ±1.65). Both of these concentrations of carvacrol (i.e. 2.5 and 5.0µg/ml) were also found to be protective against the DNA damage induced by 2.5µg/ml of parathion. Mean values of TM 4.43µM ±1.30 and 1.24µM ±1.0 observed for 2.5 and 5.0µg/ml of carvacrol respectively were significantly (P<0.05) lower than the mean TM value (6.73µM ±1.23) of PBL treated with 2.5µg/ml of parathion only. This reduction in TM value with carvacrol against parathion suggests its antigenotoxic properties.

As in SCE assay, we also studied the combined antigenotoxic effect of curcumin and carvacrol against both the pesticides (malathion and parathion) by measuring reduction in TM value as compare to their separate treatment. PBL separately treated with 25µg/ml of curcumin and with 2.5µg/ml of carvacrol in presence of 30µg/ml of malathion had resulted in mean values of TM 4.72µM ±1.34 and 6.38µM ±1.55 respectively whereas combination of 25µg/ml of curcumin and 2.5µg/ml of carvacrol
against 30µg/ml of malathion had shown the mean value of TM 5.86µM ±1.73. Similarly, PBL separately treated with 50µg/ml of curcumin and with 5.0µg/ml of carvacrol in presence of 30µg/ml of malathion had shown mean values of TM 0.99µM ±0.22 and 1.02µM ±0.64 respectively. The combination of 50µg/ml of curcumin and 5.0µg/ml of carvacrol against 30µg/ml of malathion enhanced the mean value of TM frequency to 1.93µM ±0.67.

Combinatorial antigenotoxic effect of curcumin and carvacrol was also analyzed against parathion. PBL treated with 10µg/ml of curcumin and with 2.5µg/ml of carvacrol separately in presence of 2.5µg/ml of parathion had shown mean values of TM 5.23µM ±2.29 and 4.43µM ±1.30 respectively whereas mean TM value had been increased to 5.67µM ±1.29 by combination of 10µg/ml of curcumin and 2.5µg/ml of carvacrol. Similarly, treatment of PBL with 15µg/ml of curcumin and with 5.0µg/ml of carvacrol separately in presence of 2.5µg/ml of parathion had resulted in mean values of TM 2.65µM ±1.70 and 1.24±1.01 respectively. The combination of 15µg/ml of curcumin and 5.0µg/ml of carvacrol against 2.5µg/ml of parathion enhanced the mean value of TM to 2.21µM ±0.43. We did not observe any reduction in TM value with combination of curcumin and carvacrol as compare to their separate treatment against malathion and parathion. Instead, there was small increase in TM value. However, this small increase was fond to be non significant.

**Effect of GSTM1 and GSTT1 polymorphism on genotoxicity induced by malathion and parathion and antigenotoxicity of curcumin and carvacrol**

GST is the second most studied enzyme family of multifunctional dimeric proteins which play a central role in phase II metabolism of xenobiotic compounds, in which they catalyze the conjugation of various substrates with electrophilic moieties to glutathione. The resulting more water soluble conjugates are excreted via the multidrug resistance protein efflux pumps or undergo further metabolism to mercapturic acids. Any polymorphisms that affect xenobiotics metabolism or cellular response to DNA damage could modify individual sensitivity to genotoxins. Polymorphism in *GSTM1* (mu) and *GSTT1* (theta), members of the glutathione S-transferase multigene family is the most extensively studied among human population with major ethnic differences. The homozygous *GSTM1* and *GSTT1* null genotypes have gained attention because their
frequency varies from 45% to 60% and 16% to 38% in different population. The GSTM1 and GSTT1 are candidate cancer predisposing genes because null genotype of both genes have been found associated with cancer of lung, breast, colon, stomach, bladder etc.

We studied the antigenotoxic potential of curcumin and carvacrol against the genotoxicity of both the pesticides (malathion and parathion) using SCE and comet assay as a biomarker of DNA damage and their relationship with GSTT1 and GSTM1 genetic polymorphism. Individuals having different genotypes respond differently to environmental chemicals. Multiplex PCR was used to detect the presence or absence of GSTM1 and GSTT1 genotypes. Malathion and parathion induced the DNA damage in both null and non null GSTT1 and GSTM1 genotypes. The extent of DNA damage induced by malathion and parathion in non null GSTT1 and GSTM1 genotypes was more but it was found to be non significant (P>0.05). Reduction in DNA damage by curcumin and carvacrol against malathion and parathion was more in GSTT1 and GSTM1 non null genotypes as compare to GSTT1 and GSTM1 null genotypes but that reduction was found to be statistically non significant (P>0.05).

Overall, we observed that individuals which are non-null for either GSTM1 or GSTT1 gene have reduced TM value and SCE frequency in presence of malathion and parathion as compared to those which are null for either GSTM1 or GSTT1 gene. However, reduction in genotoxicity was not found to be significant (P<0.05). Similarly, curcumin and carvacrol were found to be more antigenotoxic as measured by reduced TM value and SCE frequency in individuals having either GSTM1 or GSTT1 non-null genotypes as compared to those having GSTM1 and GSTT1 null genotypes. However, these antigenotoxic effects were not found to be statistically significant (P>0.05).

Salient findings of present investigation are summarized below -

- Both the pesticides i.e. Malathion and Parathion were found to be genotoxic to cultured human PBL at the concentrations of 30 and 2.5µg/ml respectively.
Both curcumin and carvacrol were analyzed for any genotoxicity at the concentrations used to study their antigenotoxic effect. None of these were found to be genotoxic.

Antigenotoxic effect of curcumin was analyzed by reduction in DNA damage in presence of malathion. Curcumin at the concentrations of 25 and 50µg/ml had significantly reduced DNA damage as compared to malathion treatment.

We observed that 10 and 15µg/ml of curcumin had significantly reduced the genotoxic damage caused by parathion which supports its antigenotoxic property.

Antigenotoxic effect of carvacrol was also analyzed against DNA damage induced by malathion and parathion. Carvacrol had shown protective effect against malathion and parathion at 2.5 and 5.0µg/ml.

Curcumin and carvacrol had shown more antigenotoxic effect at their higher concentrations (50µg/ml and 5.0µg/ml respectively) as compare to their lower concentrations (25µg/ml and 2.5µg/ml respectively).

We observed the small increase or decrease in SCE frequency and TM value by the combination of curcumin and carvacrol as compared to their separate treatment. However, this small increase or decrease in SCE frequency and TM value was not found to be significant (P>0.05).

GSTM1 and GSTT1 polymorphisms were not found to be significantly associated with malathion and parathion induced genotoxicity.

Our study supports the genotoxicity of malathion and parathion. There is need of steps to be taken against the usage of pesticides in fields like proper safety measures and they must be replaced with some safer analogues.
Our findings suggest that curcumin and carvacrol may be administered in diet as a preventive measure against common genotoxicants and may further be used for the development of safer medication against impacts of carcinogens.