REVIEW OF LITERATURE

2.1. Pesticides identity

A pesticide is a substance or mixture of substances used to kill a pest. A pesticide may be a chemical substance, biological agent (such as a virus or bacteria), antimicrobial, disinfectant or device used against any pest. The Food and Agriculture Organization (FAO) has defined the term of pesticide as any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term pesticides includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thining fruit or preventing the premature fall of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport (FAO, 2003). Pests include insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes (roundworms) and microbes which compete with humans for food, destroy property, spread or are a vector for disease or cause a nuisance (Agrawal and Sharma, 2010).

2.1.1. Human exposure to pesticides

Pesticides can be found, often in small amounts, almost anywhere worldwide. High exposure concerns professional use of pesticides, in the production and packaging of these compounds, or during their use in agriculture and public health. In addition to use in agriculture and forestry, pesticides are used in many public places, including office buildings, restaurants, schools, parks, golf courses, and along roads, railroads, and power lines. A great number of pesticide compounds have been found to contaminate water resources, ambient air, fog, rain, and soils in numerous studies. Most nonoccupational exposure comes from food or home pesticide use, such as pet treatments, extermination of household pests, removal of lice, and garden and lawn treatments. There are three main routes of exposure: oral, dermal, and inhalation. Ingestion of food or water containing pesticides is oral exposure. Inhalation exposure can occur by breathing air containing pesticides as vapor, aerosol, or small particles. Dermal exposure occurs when the skin comes in contact with pesticides (Bolognesi and Merlo, 2011).

2.1.2. Classification of pesticides

Table, (1): Classification of pesticides according to pest types. Table was obtained from United States Environmental Protection Agency (USEPA), 2005.

Pesticides	Use
Algicides	Control algae in lakes, canals, swimming pools, water tanks, and other sites.
Antifouling agents	Kill or repel organisms that attach to underwater surfaces, such as boat bottoms.
Antimicrobials	Kill microorganisms (such as bacteria and viruses).
Attractants	Attract pests (for example, to lure an insect or rodent to a trap). (However, food is not considered a pesticide when used as an attractant.)
Biopesticides	Biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals.
Biocides	Kill microorganisms.
Disinfectants and sanitizers	Kill or inactivate disease-producing microorganisms on inanimate objects.
Fungicides	Kill fungi (including blights, mildews, molds, and rusts).
Fumigants	Produce gas or vapor intended to destroy pests in buildings or soil.
Herbicides	Kill weeds and other plants that grow where they are not wanted.
Insecticides	Kill insects and other arthropods.
Miticides (also called acaricides)	Kill mites that feed on plants and animals.
Microbial pesticides	Microorganisms that kill, inhibit, or out compete pests, including insects or other microorganisms.
Molluscicides	Kill snails and slugs.
Nematicides	Kill nematodes (microscopic, worm-like organisms that feed on plant roots).
Ovicides	Kill eggs of insects and mites.
Pheromones	Biochemicals used to disrupt the mating behavior of insects.
Repellents	Repel pests, including insects (such as mosquitoes) and birds.
Rodenticides	Control mice and other rodents.
Defoliants	Cause leaves or other foliage to drop from a plant, usually to facilitate harvest.
Desiccants	Promote drying of living tissues, such as unwanted plant tops.
Insect growth regulators	Disrupt the molting, maturity from pupal stage to adult, or other life processes of insects.
Plant growth regulators	Substances (excluding fertilizers or other plant nutrients) that alter the expected growth, flowering, or reproduction rate of plants.

2.1.3. Link between pesticide exposure and incidence of chronic diseases

Chronic diseases are characterized by their generally slow progression and long term duration, which are considered as the leading cause of mortality in the new world (Mostafalou and Abdollahi, 2013).

2.1.3.1. Cancer

During the past half century, a wide spectrum of population-based studies has been carried out in this respect leading to a significant progress in understanding the relationship of pesticides to the incidence of different types of malignancies (Penel and Vansteene, 2007). The International Agency for Research on Cancer (IARC) has conducted several cohort studies on the incidence of cancers in people exposed to pesticides somehow during their lives (Baldi and Lebailly, 2007).

2.1.3.2. Birth defects

Birth defects or congenital disorders are defined as structural or functional abnormalities existing at birth or before birth that causes physical or mental disabilities. Ranging from mild to fatal, diverse types of birth defects have been recognized and deliberated as the principal cause of death for infants during the first years of life. Any material which can induce birth defects is called teratogen (Rogers and Kavlock, 2008).

2.1.3.3. Chronic respiratory disease

Asthma is considered as the most common disorder among chronic respiratory dysfunctions affecting both children and adults. Its close relationship with work-related exposures has been known from eighteen centuries so that occupational asthma is characterized as a disease in medicine. There have been several reports on increased rate of asthma in people occupationally exposed to pesticides (Hernandez *et al.*, 2011).

2.1.3.4. Chronic nephropathies

Higher incidence of the late-onset nephropathies like chronic kidney disease and chronic renal failure has been reported in middle-aged people (40–60 years) living in the agricultural areas with more prevalence in men. The results of a survey in North Central Province of Sri Lanka have presented a significant relationship between chronic renal failure and environmental factors in farming areas (Wanigasuriya *et al.*, 2007). Exposure to acetylcholinesterase inhibiting pesticides was associated with chronic renal failure (Peiris-John *et al.*, 2006).

2.1.3.5. Other chronic diseases

However, there are sporadic reports on the association of exposure to pesticides with different types of human chronic diseases, including chronic fatigue syndrome (Behan and Haniffah, 1994), autoimmune diseases like systemic lupus erythematous and rheumatoid arthritis (Parks *et al.*, 2011).

2.2. *Bacillus thuringiensis*

2.2.1. The *Bacillus* taxonomy

The family *Bacillaceae* is a highly diverse taxonomic group of endospore-forming bacteria, with the two main genera *Clostridium* and *Bacillus*. The bacteria within the genus *Clostridium* are Gram positive and anaerobic and a few species are often associated with well known human diseases; botulism (*Clostridium botulinum*), tetanus (*Clostridium tetani*), food poisoning and gas-gangrene (*Clostridium perfringens*). The genus *Bacillus* is composed of aerobic or facultative anaerobic Gram-positive bacteria. With the exceptions of *Bacillus mycoides* and *Bacillus anthracis*, *Bacillus* species are generally motile. Under specific growth conditions a few species; *Bacillus anthracis*, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus megateriumcan* also produce a protective polypeptide capsule (poly-γ-D-glutamic acid) (Turnbull and Kramer, 1995). The majority of *Bacillus* species are rarely associated with human diseases with a few exceptions within the *Bacillus cereus* and *Bacillus subtilis* groups (Drobniewski, 1993).

2.2.2. The *Bacillus cereus* group

The *Bacillus cereus* group is composed of the species; *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*. In addition; the rhizoid-forming *Bacillus mycoides*, *Bacillus pseudomycoides* and the psychrotoloant *Bacillus weihenstephanensis* have been proposed to belong to the *Bacillus cereus* group (Lechner *et al.*, 1998; Nakamura, 1998).

Bacillus anthracisis a highly monomorphic species within the *Bacillus cereus* group, showing very little genetic variation (Keim *et al.*, 1997). In the environment, the bacterium primarily exists as a highly stable, dormant spore in the soil. Nevertheless, it has been claimed that the organism can grow and persist outside the host, in the rhizosphere of plants (Saile and Koehler, 2006). *Bacillus anthracisis* the cause of anthrax, primarily a disease of herbivores, but may also cause isolated cases of infections in man. Anthrax takes three forms: cutaneous, gastro-intestinal, or inhalational (Mock and Fouet, 2001). While the cutaneous form is easily treatable with antimicrobials, the gastrointestinal and inhalational forms of the disease are more severe, as has been demonstrated by human deaths following the ingestion of meat from animals that died from anthrax disease (Beatty *et al.*, 2003).

Bacillus cereus is a common bacterium of the soil and can colonize invertebrate guts as a symbiont, an environment which has been suggested to be its natural habitat (Jensen *et al.*, 2003). It is however a frequent cause of human food poisoning, as well as various opportunistic and nosocomial infections, e.g. in the immunocompromised or following trauma to the eye (Bottone, 2010).

Bacillus thuringiensis is also frequent in soil, is an entomopathogenic bacterium, and is the most commonly used commercial biopesticide worldwide (Soberon *et al.*, 2007). Its identification and classification are based on the production of insecticidal proteinaceous toxin crystals during sporulation (Aronson, 2002), a feature recognized by microscopy. The Cry toxins are of different classes and exhibit variable specificities towards the larvae of different classes of insects (Schnepf *et al.*, 1998).

The resilient endospores formed in this group are uniform in size, shape and spore-surface make up. The spores of the *Bacillus cereus* group are approximately 1 µm in size and well within inhalable size for mice and humans (Figure 1) (Carrera *et al.,* 2007)

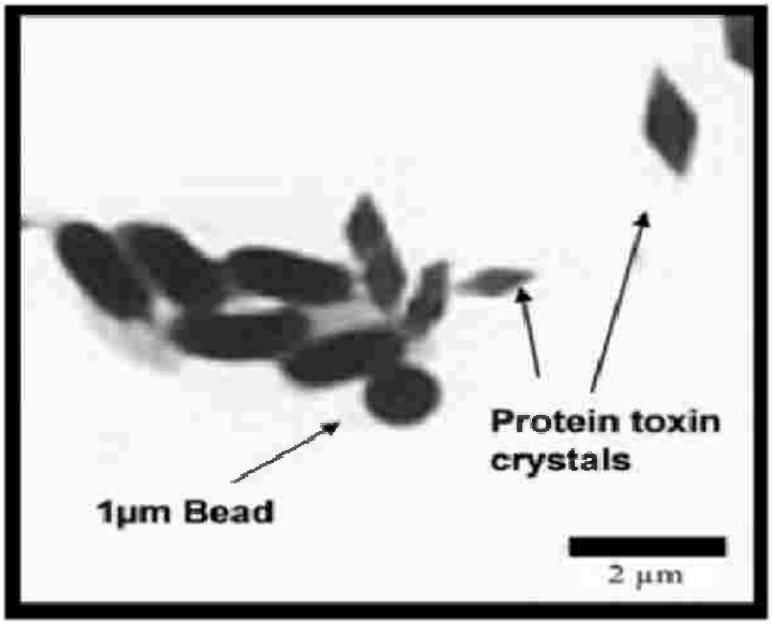

Fig. 1. Electron micrographic image of *Bacillus thuringiensis* spores and crystal toxins. Polystyrene beads (1µm diameter) were used as an internal standard and, along with the crystal toxins, are indicated by arrows in the electron micrographic images (Carrera *et al.*, 2007).

2.2.3. The insecticidal properties of *Bacillus thuringiensis*

2.2.3.1. The pore formation model:

During formation of the *Bacillus thuringiensis* endospores the crystal proteins (Cry) are secluded from the spore and toxin crystals are formed. Depending of their protein makeup, the crystal toxins agglutinate and take various forms; rhomboid, cubical, bipyramidal or a mix. Undisturbed the crystal will remain associated with the endospore within the original bacterial coat. After ingestion by the insect larvae the *Bacillus thuringiensis* spore and toxin will pass through the acidic stomach of most insects. However, under alkaline conditions in the insect mid-gut (pH 11-12) the Cry proteins are converted to pro-toxins and the insects'digestive proteases will cleave the pro-toxins into the active δ-endotoxin form (Figure 2). The δ-endotoxin binds to specific receptors on mid-gut epithelial cells. The δ-endotoxin then undergoes conformational change and is thought to act as a pore-forming toxin causing lysis of the gut cell walls. This will in turn cause gut paralysis by creating an ionic imbalance over the epithelium. Finally, the significantly lower pH in the gut and the release of nutrients into the digestive tract will create the basis for spore germination and proliferation of the bacteria. The bacterial spore thus survives the transport to the weakened insect mid-gut and is able to penetrate and multiply within the insect gut and haemocoel. The δ-endotoxins are in themselves able to kill insect larvae after ingestion, but the bacterial spore has been shown to enhance the toxic effect (Dubois and Dean, 1995). After multiplying within the dying or dead insect host, the depletion of

nutrients will lead to sporulation and eventually release of new endospores to the environment (Schnepf, 1998).

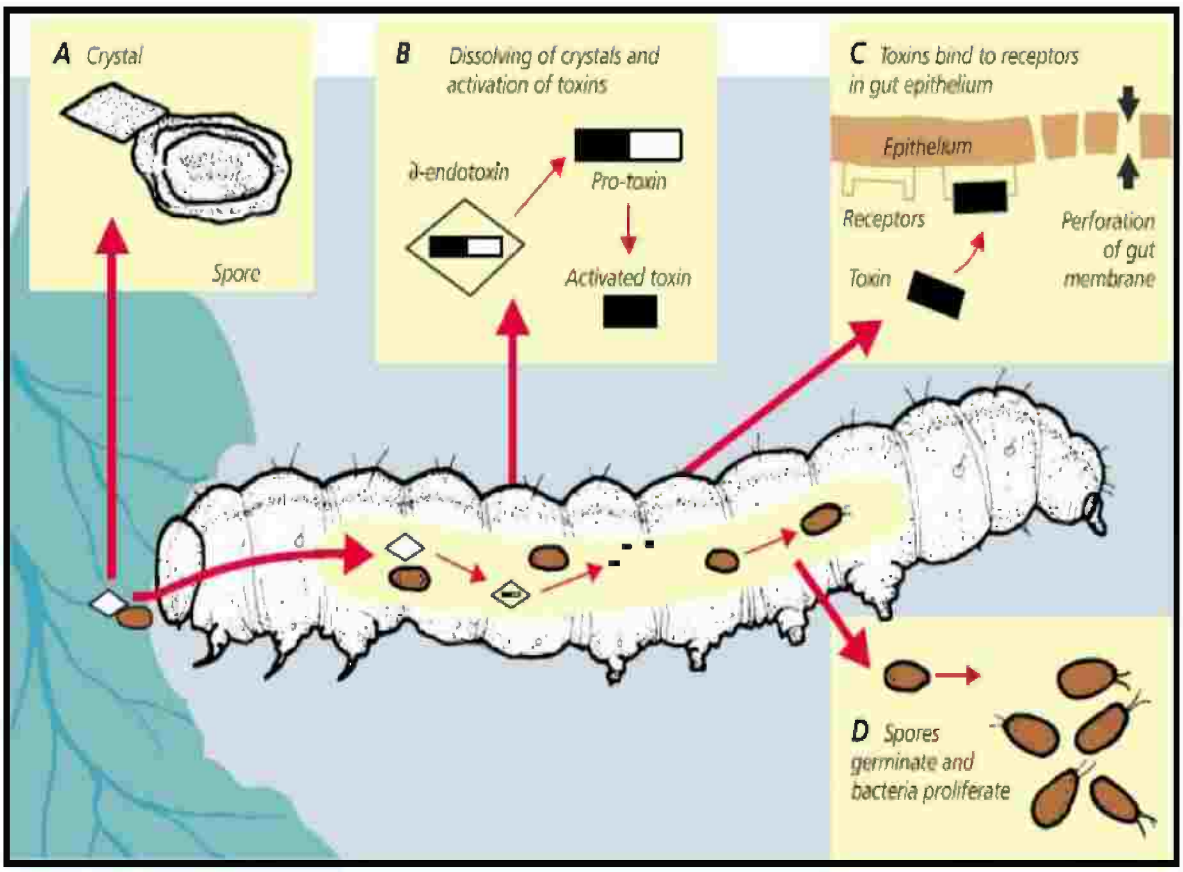

Fig. 2. Mode of action of *Bacillus thuringiensis*. **A**, Toxin crystal and spore are ingested. **B**, Activation of toxin. **C**, Binding of toxin to receptor and perforation of the gut epithelium. **D**, Germination and proliferation of bacteria (WHO, 1999).

2.2.4. Use of *Bacillus thuringiensis* in pest control

Commercial insecticides derived from this bacterium have a long history of successful use in the biocontrol of insect pests (Sanchis, 2011), in agriculture (Sanchis and Bourguet, 2008) and forestry (van Frankenhuyzen, 2000). For the last decade and a half, several transgenic crops that express insecticidal *Bt* toxins have been grown over a rapidly increasing area (James, 2010).

2.2.5. Effect *Bacillus thuringiensis* on lipid peroxidation in rat liver

Shaban *et al.* (2003) studied the effect of Dipel, a *Bacillus thuringiensis*-based biopesticide, on hepatic antioxidant enzyme activities and lipid peroxidation in rat liver. In this study, animals were treated with 1 mg/100 g body mass for 4 successive days. They found that Dipel increased the activities of glutathione peroxidase (GPx), glutathione reductase (GR) and the level ofmalondialdehyde (MDA) in rat hepatocytes. The activity of superoxide dismutase (SOD) and reduced glutathione (GSH) level were decreased. The results indicated that Dipel induced oxidative stress in rat liver.

2.2.6. Effect *Bacillus thuringiensis* on fertility and organ toxicity

Lemos *et al.* (2013) studied the effect of sub-lethal doses of *Bacillus thuringiensis* subsp. *Aizawai* with regard to fertility and histopathology of the kidneys, liver and lungs as well as the morphology of the neonates. In this study pregnant albino rats were treated with 1, 10 or 20 mg of protein toxin *B. thuringiensis* subsp. *Aizawai* /100g. Anmials were administered orally for either seven days or during the entire pregnancy. They found that no miscarriages occurred and the neonates did not exhibit signs of malformation of the head, limbs, thorax or abdomen. However, there were a smaller number of pups in the groups that received higher doses of the biopesticide in comparison to the control group. Biopesticide produced lesions in the kidneys, liver and lungs and reduced the fertility of rats when administered at sub-lethal doses with no clinical signs of intoxication. Thus, this study suggested that sublethal doses of biopesticide can provide chronic toxicity in humans.

2.3. Carbamate insecticides

2.3.1. Chemical Structure of Carbamate insecticides

Carbamate pesticides are derivatives of carbamic acid, which has the structure in Figure 3 (Ware, 2000). The carbamate esters that are derived have the common structure also shown in Figure 3, where the box identifies where attachment of an alcohol, oxime or phenol would be (Baron, 1991).

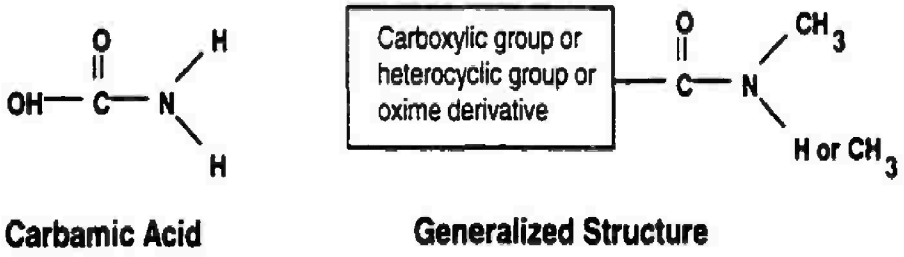

Fig. 3. General structures of carbamates (Ware, 2000; Baron, 1991)

2.3.2. Absorption, Distribution and metabolism of carbamate

2.3.2.1. Absorption of carbamate

Most carbamates are readily absorbed through the skin (dermal), respiratory tract (inhalation), and gastrointestinal tract (oral). The most important and likely human exposure route, for the general populace, is dermal, and the absorption rate is greatly influenced by the vehicle and environmental conditions. For example, the dermal absorption of methomyl is increased in conditions of high temperature and humidity. Carbamates are also readily absorbed through the gastrointestinal tract after exposure to residues in foods (Baron, 1991).

2.3.2.2. Distribution of carbamate

Once absorbed, distribution of carbamates is rapid to the tissues and organs of the body. Tissues and organs that are responsible for xenobiotic metabolism tend to have the highest concentrations of carbamates (Baron, 1991).

2.3.2.3. Metabolism of carbamate

The first step in carbamate metabolism is oxidation (N-demethylation, aromatic ring hydroxylation, O-dealkyiation, alkyl hydroxylation, and sulfoxidation) that provides a site for a conjugation reaction (O- and N-glucuronides. sulfates, and mercapturic acid derivatives) yielding water-soluble products for excretion (Baron, 1991). Oxidative reactions can be classified into two groups:

1. Ring hydroxylation that may sometimes further oxidize to ketones or epoxidation followed by hydrolysis to the corresponding diol.

2. Oxidation of side-chains. The side chain oxidations can take on many forms, and various aliphatic ring substitutions can be hydroxylated. Methyl groups can become hydroxymethyls and isopropyl moieties can become l- hydroxyisopropyl groups. *N*-demethylation is extremely important for carbamates, since every carbamate posses an *N*-alkyl group. *N*- hydroxymethylation may take place by stepwise reactions, but some carbamate molecules possess thioethers in side chains that can be oxidized to sulfoxides and sulfones.

Regardless of the oxidative metabolic activities for carbamate insecticides, they can cause a variety of changes that will influence their toxicity and residual characteristics. Oxidized biotransformation products can be either more toxic or less toxic than the parent compound, but they usually do not become completely nontoxic.

Generally, oxidation results in decreased stability of the molecule and provides sites for attack by conjugative enzymes so that although oxidation can result in activation, the net result is to increase overall levels of detoxification: however, whether there is activation or detoxification can be judged only by the stability, availability, and frequency of appearance of each metabolite in the animal.

There are also two esterase-mediated routes of carbamate metabolism that have been shown: 1) esterases attack the bond on the side of the carbonyl group attached to the oxygen.

2) Amidases attack the bond on the side attached to the nitrogen atom. Esterase-catalyzed hydrolysis is probably less important for carbamates relative to oxidative metabolism, although the extent to which hydrolysis occurs depends on both the type of carbamate and organism (Perry *et al.*, 1998).

2.3.3. Mechanism of action

Carbamate pesticides cause reversible carbamaylation of the acetylcholinesterase enzyme, allowing accumulation of acetylcholine, the neuromediator at parasympathetic neuro-effector junctions (muscarinic effect) and autonomic gangli (nicotinic effect) and in the brain (central nervous system effect) (Mahgoub and Mednay, 2001).

2.3.3.1. Enzyme Inhibition Kinetics

The active site of acetylcholinesterase (AChE) contains two sites of ligand binding, an acylation site and a peripheral site. If the enzyme (E) reacts with an inhibitor (AX) in an initially reversible manner, the reaction can be represented by

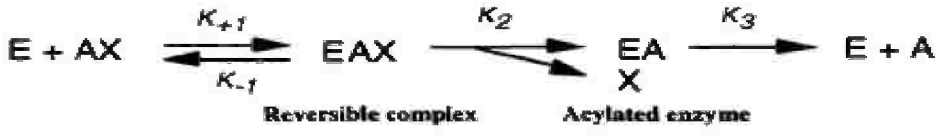

Fig. 4. Representation of the enzyme-inhibitor complex (Rosenberry *et al.*, 1999)

The equation in Figure 4 is very similar to the traditional AChE catalytic pathway (Figure 5), which shows the initial enzyme-substrate complex ES (EAX. Figure 4)

proceeding to an acylated enzyme intermediate EA (EAX, Figure 4) and then hydrolyzed to products P and E (E + A, Figure 4) (Rosenberry *et al.*, 1999).

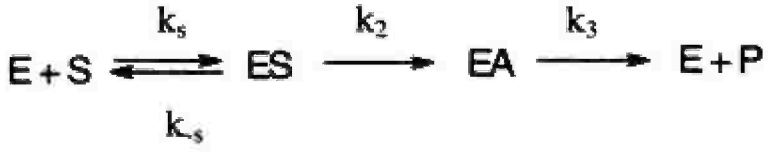

Fig. 5. Traditional AChE catalytic pathway (Perry *et al.*, 1998).

In the equation (figure 4), EAX represents the enzyme-inhibitor complex, X is the leaving group, A is that part of the inhibitor molecule that eventually blocks the enzyme, and EA is the inhibited enzyme or acylated enzyme intermediate. The affinity with which the substrate binds to the enzyme in the first step is often described by the dissociation constant (Perry *et al.*, 1998).

$$K_d = k_{-1}/k_{+1} = \frac{[E][AX]}{[EAX]}$$

Fig. 6. Equation describing the dissociation constant (K_d) for the substrate-enzyme binding (Perry *et al.*, 1998)

The smaller K_d, the greater the affinity of the enzyme for the inhibitor. In Figure 4, the rate constant K_2 defines the acylation step, and K_3 defines the rate of enzyme recovery. The rate at which the enzyme is inhibited, or the rate at which EA is formed, is defined by two different processes: 1) affinity of the enzyme for the inhibitor or K_d and 2) the rate at which the enzyme becomes acylated or K_2 (Perry *et al.*, 1998).

A measure that takes into account the contributions of both K_d and K_2 is the inhibition constant, or bimolecuiar rate constant (K_i). This constant is defined as $K_i = K_2/K_d$. The bimolecular rate constant is particularly useful for comparing the inhibition potency of various compounds (i.e. comparing the sensitivity of AChE) to inhibition between resistant and susceptible strains of insects (Perry *et al.*, 1998).

Carbamate pesticides produce toxic effects by inhibiting AChE (Perry *et al.*, 1998). Inhibition results from the formation of a carbamate-AChE complex (carbamylation of AChE) (Baron, 1991).

2.3.4. Methomyl

Methomyl [IUPAC: S-methyl N-(methylcarbamoyloxy) thioacetimidate] is a commonly used monomethyl carbamate insecticide (Clive, 2001) to control a wide range of insects and spider mites through direct contact and ingestion (WHO, 1996).

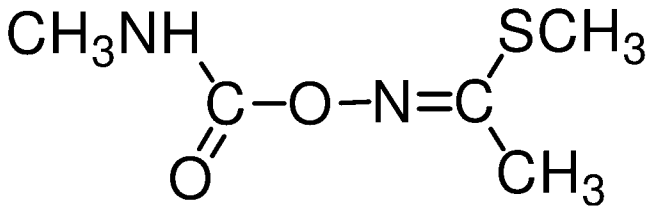

Fig. 7. Chemical structure of methomyl (WHO, 1996)

2.3.4.1. Toxiclogical effects of methomyl

2.3.4.1.1. Acute toxicity

Methomyl has a high mammalian toxicity, the acute oral LD_{50} for male rats is 34 mg/ kg and for female rats it is 30 mg/kg (Tomlin, 2006). Symptoms of methomyl exposure may include weakness, blurred vision, headache, abdominal cramps, chest discomfort, constriction of pupils, sweating, muscle tremors and decreased pulse. If there is severe poisoning, symptoms of twitching, giddiness, confusion, muscle incoordination, slurred speech, low blood pressure, heart irregularities and loss of reflexes may be experienced. Death can result from discontinued breathing, paralysis of muscles of respiratory system, intense constriction of the openings of lung, or all three (Baron,1991).

2.3.4.1.2. Chronic toxicity

Prolonged or repeated exposure to methomyl may cause symptoms similar to the pesticide acute effects. Repeated exposure to small amounts of methomyl may cause an unsuspected inhibition of cholinesterase, resulting in flu-like symptoms, such as weakness, lack of appetite and muscle aches. Cholinesterase-inhibition may persist for two to six weeks. This condition is reversible if exposure is discontinued. Since cholinesterase is increasingly inhibited with each exposure, severe cholinesterase- inhibition symptoms may be produced in a person who has had pervious methomyl exposure, while a person without previous exposure may not experience any symptoms at all (Baron,1991). In a 24 mouth study with rats fed doses of 2.5,5 or 20 mg/kg/day, effects were only observed at the highest dose tested, 20mg/kg/ day. At this very high dose, red blood cell counts and hemoglobin levels were significantly reduced in female rats (USEPA, 1987).

2.3.4.1.3. Hepatic and renal toxicity

The study by El-Demerdash *et al.* (2012) was designed to evaluate the toxic effects induced by different time intervals of methomyl exposure on liver antioxidant defense system, oxidative stress and liver function biomarkers in CD-1 mice. In this study anmials were orally treated with one mg methomyl/kg BW for 10, 20 and 30 days, respectively. They reported that methomyl significantly induced TBARS and decreased the activity of antioxidant enzymes, glutathione S-transferase, superoxide dismutase and catalase and the levels of reduced glutathione in mice liver. Aminotransferases and alkaline phosphatase activities were significantly decreased in liver due to methomyl administration, while the activities of these enzymes were significantly increased in serum. In addition, liver lactate dehydrogenase activity was significantly increased. On the contrary, methomyl treatment caused a significant decrease in liver acid phosphatase. Results concluded that exposure to

methomyl induced toxicity and oxidative stress in mice liver via free radicals mechanism. Also, methomyl might have affected cell metabolism, cell membrane permeability and the detoxification system in liver.

The study by El-Demerdash *et al.* (2013a) was carried out to investigate the effects of methomyl at different time intervals on lipid peroxidation, GSH, total sulfhydryl group (T-SH), antioxidant enzymes such SOD, CAT and glutathione S-transferase (GST) in mice kidney. In this study animals were orally treated with one mg methomyl/kg BW for 10, 20 and 30 days, respectively. They found that methomyl significantly increased lipid peroxidation in kidney as compared to control group. Levels of GSH and T-SH and activities of SOD, CAT and GST were found to be decreased. On the other hand, methomyl significantly increased the levels of urea, uric acid and creatinine in serum. The results of this study suggested that methomyl exposure can cause renal damage, oxidative stress, perturbations in antioxidant defense system in mice kidney in a time dependent manner.

The study by Djeffal *et al.* (2015) was reported that methomyl -induced changes in biochemical markers and oxidative damage in blood, liver and kidney of male Wistar rats. Animals were received MET (8 mg/kg body weight) in drinking water for 21 days. A significant increase in the levels of hepatic markers enzymes alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase was determined. Furthermore, renal markers such as urea and creatinine were increased in MET-treated rats. Exposure of rats to MET caused significant increase in malondialdehyde levels, thus causing a drastic alteration in antioxidant defense system, particularly in the activities of CAT and GST and GP_X. The results of this study revealed that MET-induced toxicity caused perturbations of some biochemical parameters, lipid peroxidation and alterations in the antioxidant enzymes in liver and kidney homogenates.

2.3.4.1.4. Reproductive toxicity

The reproductive toxicity of methomyl in male rats was manifested by lowered fertility index, decreased weight of the testes, seminal vesicles and prostate glands and lowered semen quantity and quality (Shalaby *et al.*, 2010) and Mahgoub and Mednay (2001) reported that hormonal changes and testicular damage after chronic exposure of male rats to insecticide methomyl. The authors concluded that methomyl has deleterious effects on rat testes. Other carbamate insecticides were found to adversely affect male reproductive organs and semen quality. Pant *et al.* (1995) reported a significant decrease in the weight of the testes, seminal vesicles and prostate glands caused by carbamate insecticide carbofuran. Pant *et al.* (1996) mentioned that the carabamate insecticide carbaryl had no significant changes in the weight of testes, epididymides and accessory sex organs, but was spermiotoxic in the rats. In addition, Pant *et al.* (1997) found that carbofuran insecticide in adult and young male rats caused significant decreases in epididymal sperm count and sperm motility, with an increase in sperm abnormal morphology.

2.4. Oxidative stress

2.4.1. Definition of Reactive oxygen species (ROS)

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell and Gutteridge, 1999). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller *et al.*, 1990). Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism. At low to moderate concentrations, they function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components, such as lipids, proteins, and DNA (Halliwell and Gutteridge, 1999; Marnett, 1999).

The shift in balance between oxidant/antioxidant in favor of oxidants is termed "oxidative stress". Oxidative stress contributes to many pathological conditions, including diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease (Asami *et al.,* 1997) and asthma (Fitzpatrick *et al.*, 2009).

2.4.2. Endogenous sources of ROS

ROS are produced from molecular oxygen as a result of normal cellular metabolism. ROS can be divided into 2 groups: free radicals and nonradicals. Molecules containing one or more unpaired electrons and thus giving reactivity to the molecule are called free radicals. When two free radicals share their unpaired electrons, nonradical forms are created. The three major ROS that are of physiological significance are superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) (Birben *et al.*, 2012). ROS are summarized in Table 2.

Table (2): Major endogenous oxidants (Birben *et al.*, 2012)

Oxidant	Formula	Reaction Equation
Superoxide anion	$O_2^{-\cdot}$	• $NADPH+2O_2 \rightarrow NADP^+ + 2\ O_2^{-\cdot} + H^+$
Hydrogen peroxide	H_2O_2	• $2O_2^{-\cdot} + H \rightarrow O_2 + H_2O_2$ • $Hypoxanthine + H_2O + O_2 \leftrightarrow xanthine + H_2O_2$ • $Xanthine + H_2O + O_2 \leftrightarrow uric\ acid + H_2O_2$
Hydroxyl radical	$^{\cdot}OH$	• $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$
Hypochlorous acid	HOCl	• $H_2O_2 + Cl^- \rightarrow HOCl + H_2O$
Peroxyl radicals	$ROO^{\cdot}$	• $R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$
Hydroperoxyl radical	$HOO^{\cdot}$	• $O_2^- + H_2O \leftrightarrow HOO^{\cdot} + OH$

Superoxide anion is formed by the addition of one electron to the molecular oxygen (Miller *et al.*, 1990).This process is mediated by nicotine adenine dinucleotide phosphate (NADPH) oxidase or xanthine oxidase or by mitochondrial electron transport system. The major site for producing superoxide anion is the mitochondria, the machinery of the cell to produce adenosine triphosphate.

Hydrogen peroxide easily diffuses across the plasma membrane. Hydrogen peroxide is also produced by xanthine oxidase, amino acid oxidase, and NADPH oxidase (Dupuy *et al.*, 1991) and in peroxisomes by consumption of molecular oxygen in metabolic reactions. In a succession of reactions called Haber–Weiss and Fenton reactions, H_2O_2 can breakdown to OH^- in the presence of transmission metals like Fe^{2+}or Cu^{2+}(Fenton,1984)

$$Fe^{3+} + {}^{\cdot}O_2 \rightarrow Fe^{2+} + O_2 \quad \text{Haber-Weiss}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \quad \text{Fenton reaction}$$

O_2^- itself can also react with H_2O_2 and generate OH^- (Liochev and Fridovich, 2002). Hydroxyl radical is the most reactive of ROS and can damage proteins, lipids, and carbohydrates and DNA. It can also start lipid peroxidation by taking an electron from polyunsaturated fatty acids.

Granulocytic enzymes further expand the reactivity of H_2O_2 via eosinophil peroxidase and myeloperoxidase (MPO). In activated neutrophils, H_2O_2 is consumed by MPO. In the presence of chloride ion, H_2O_2 is converted to hypochlorous acid (HOCl). HOCl is highly oxidative and plays an important role in killing of the pathogens in the airways (Klebanoff, 2005). However, HOCl can also react with DNA and induce DNA–protein interactions and produce pyrimidine oxidation products and add chloride to DNA bases (Kulcharyk and Heinecke, 2001).

Other oxygen-derived free radicals are the peroxyl radicals ($ROO^{\cdot}$). Simplest form of these radicals is hydroperoxyl radical ($HOO^{\cdot}$) and has a role in fatty acid peroxidation. Free radicals can trigger lipid peroxidation chain reactions by abstracting a hydrogen atom from a side chain methylene carbon. The lipid radical then reacts with oxygen to produce peroxyl radical. Peroxyl radical initiates a chain reaction and transformspolyunsaturated fatty acids into lipid hydroperoxides. Lipid hydroperoxides are very unstable and easily decompose to secondary products, such as aldehydes (such as 4-hydroxy-2,3-nonenal) and malondialdehydes (MDAs). Isoprostanes are another group of lipid peroxidation products that are generated via the peroxidation of arachidonic acid and have also been found to be elevated in plasma and breath condensates of asthmatics (Wood *et al.*, 2000).

2.4.3. Exogenous source of oxidants

The exogenous sources of oxidants include ozone exposure, ionizing radiation, heavy metal ions and pesticides.

2.4.3.1. Ozone exposure

Ozone exposure can cause lipid peroxidation and induce influx of neutrophils into the airway epithelium. Short-term exposure to ozone also causes the release of inflammatory mediators, such as MPO, eosinophil cationic proteins and also lactate dehydrogenase and albumin (Hiltermann *et al.*, 1999).

2.4.3.2. Ionizing radiation

Ionizing radiation, in the presence of O_2, converts hydroxyl radical, superoxide, and organic radicals to hydrogen peroxide and organic hydroperoxides. These hydroperoxide species react with redox active metal ions, such as Fe and Cu, via Fenton reactions and thus induce oxidative stress (Biaglow *et al.*, 1992).

2.4.3.3. Heavy metal ions

Heavy metal ions, such as iron, copper, cadmium, mercury, nickel, lead, and arsenic, can induce generation of reactive radicals and cause cellular damage via depletion of enzyme activities through lipid peroxidation and reaction with nuclear proteins and DNA (Stohs and Bagchi, 1995).

2.4.3.4. Pesticides

There is a huge body of literature on induction of oxidative stress by pesticides, and it has been implicated in development of health problems mediated by exposure to pesticides (Grosicka-Maciag, 2011; Slaninova *et al.*, 2009). It has been revealed that pesticides can disturb oxidative homeostasis through direct or indirect pathways, including mitochondrial or extramitochondrial production of free radicals, thiol oxidation, and depletion of cellular antioxidant reservoirs (Abdollahi *et al.*, 2004).

2.4.4. Enzymatic Antioxidants

The major enzymatic antioxidants are SODs (EC 1.15.1.11), CAT (EC 1.11.1.6), and GPx (EC 1.11.1.9). In addition to these major enzymes, other antioxidants, including heme oxygenase-1 (EC 1.14.99.3), and redox proteins, such as thioredoxins (TRXs, EC 1.8.4.10), peroxiredoxins (PRXs, EC 1.11.1.15), and glutaredoxins, have also been found to play crucial roles in the antioxidant defenses (Birben *et al.*, 2012).

Table (3): Enzymatic scavenger of antioxidant defense (Birben *et al.*, 2012).

Name of Scavenger	Acronym	Catalyzed Reaction
Superoxide dismutase	SOD	• $M^{(n+1)+}$-SOD+O_2^-→ M^{n+}-SOD +O_2 • M^{n+}-SOD+O_2^-+2H^+→$M^{(n+1)}$-SOD+H_2O_2
Catalase	CAT	• 2 H_2O_2→O_2+2H_2O
Glutathione peroxidase	GPx	• 2GSH+H_2O_2→GSSG+2H_2O • 2GSH+ROOH→GSSG+ROH+H_2O
Thioredoxin	TRX	• Adenosinemonophosphate+sulfite +thioredoxindisulfide=5'-adenylyl sulfate + thioredoxin
Peroxiredoxin	PRX	2 R'-SH +ROOH=R'-S-S-R'+H_2O+ROH
Glutathione S-transferase	GST	RX+GSH=HX+R-S-GSH

Since superoxide is the primary ROS produced from a variety of sources, its dismutation by SOD is of primary importance for each cell. All three forms of SOD, that is, CuZnSOD, Mn-SOD, and EC-SOD. Mn-SOD is localized in the mitochondria matrix. EC-SOD is primarily localized in the extracellular matrix, especially in areas containing high amounts of type I collagenfibers and around pulmonary and systemic vessels. It has also been detected in the bronchial epithelium, alveolar epithelium, and alveolar macrophages (Kinnula and Crapo, 2003). Overall, CuZnSOD and Mn-SOD are generally thought to act as bulk scavengers of superoxide radicals.

H_2O_2 that is produced by the action of SODs or the action of oxidases, such as xanthine oxidase, is reduced to water by catalase and the GPx. Catalase exists as a tetramer composed of four identical monomers, each of which contains a heme group at the active site. Degradation of H_2O_2 is accomplished via the conversion between two conformations of catalase-ferricatalase (iron coordinated to water) and iron complexed with an oxygen atom. Catalase also binds NADPH as a reducing equivalent to prevent oxidative inactivation of the enzyme by H_2O_2 as it is reduced to water (Kirkman *et al.*, 1999).

Enzymes in the redox cycle responsible for the reduction of H_2O_2 and lipid hydroperoxides (generated as a result of membrane lipid peroxidation) include the GPxs (Flohé, 1988). The GPxs are a family of tetrameric enzymes that contain the unique amino acid selenocysteine within the active sites and use low-molecular-weight thiols, such as GSH, to reduce H_2O_2 and lipid peroxides to their corresponding alcohols. In addition, disposal of H_2O_2 is closely associated with several thiol-containing enzymes, namely, TRXs (TRX1 and TRX2), thioredoxin reductases (EC 1.8.1.9), PRXs (which are thioredoxin peroxidases), and glutaredoxins (Gromer *et al.*, 2004).

GSTs (EC 2.5.1.18), another antioxidant enzyme family, inactivate secondary metabolites, such as unsaturated aldehydes, epoxides, and hydroperoxides. Three major families of GSTs have been described: cytosolic GST, mitochondrial GST (Robinson *et*

al., 2004) and membrane-associated microsomal GST that has a role in eicosanoid and GSH metabolism (Jakobsson *et al.*, 1999).

2.4.5. Nonenzymatic Antioxidants

Nonenzymatic antioxidants include low-molecular-weight compounds, such as vitamins (vitamins C and E), β-carotene, and GSH, a tripeptide (L-g-glutamyl-L-cysteinyl-Lglycine) that comprise a thiol (sulfhydryl) group (Birben *et al.*, 2012)

2.4.5.1. Ascorbic Acid (Vitamin C)

Water-soluble vitamin C provides intracellular and extracellular aqueous-phase antioxidant capacity primarily by scavenging oxygen free radicals (Bunker, 1992; Mezzetti *et al.*, 1996).

2.4.5.2. α-Tocopherol (Vitamin E)

Lipid-soluble vitamin E is concentrated in the hydrophobic interior site of cell membrane and is the principal defense against oxidant-induced membrane injury. Vitamin E donates electron to peroxyl radical, which is produced during lipid peroxidation. Vitamin E triggers apoptosis of cancer cells and inhibits free radical formations (White *et al.*, 1997).

2.4.5.3. Glutathione

Reduced glutathione (GSH) is highly abundant in all cell compartments and is the major soluble antioxidant. The ratio of reduced GSH to oxidized GSH (GSSG) (GSH/GSSG) is a major determinant of oxidative stress. GSH shows its antioxidant effects in several ways (Masella *et al.*, 2005). It detoxifies hydrogen peroxide and lipid peroxides via action of GPx. The GSH donates its electron to H_2O_2 to reduce it into H_2O and O_2. GSSG is again reduced into GSH by GSH reductase that uses NADPH as the electron donor.

2.4.6. Effects of oxidative stress on DNA

ROS can lead to DNA modifications in several ways, which involves degradation of bases, single or doublestranded DNA breaks, purine, pyrimidine or sugar-bound modifications, mutations, deletions or translocations, and cross-linking with proteins. Most of these DNA modifications (Figure 8) are highly relevant to carcinogenesis, aging, and neurodegenerative, cardiovascular, and autoimmune diseases. Tobacco smoke, redox metals, and nonredox metals, such as iron, cadmium, chrome, and arsenic, are also involved in carcinogenesis and aging by generating free radicals or binding with thiol groups. Formation of 8-hydroxyguanosine (8-OHG) is the bestknown DNA damage occurring via oxidative stress and is a potential biomarker for carcinogenesis (Birben *et al.*, 2012).

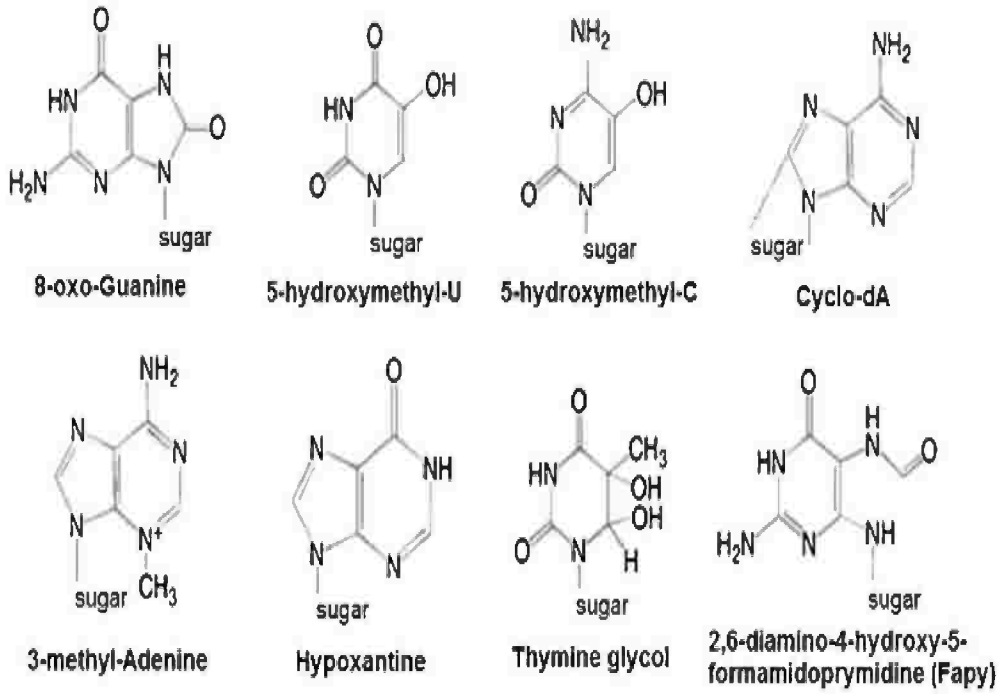

Fig. 8. Base modifications introduced by reactive oxygen species (Birben *et al*., 2012).

2.4.7. Effects of oxidative stress on lipids

ROS can induce lipid peroxidation and disrupt the membrane lipid bilayer arrangement that may inactivate membrane-bound receptors and enzymes and increase tissue permeability (Girotti, 1985). Products of lipid peroxidation, such as malondialdehyde (MDA) and unsaturated aldehydes, are capable of inactivating many cellular proteins by forming protein cross-linkages (Esterbauer *et al*., 1986).

2.4.8. Effects of oxidative stress on proteins

ROS can cause fragmentation of the peptide chain, alteration of electrical charge of proteins, cross-linking of proteins and oxidation of specific amino acids and therefore lead to increased susceptibility to proteolysis by degradation by specific proteases (Kelly and Mudway, 2003). Cysteine and methionine residues in proteins are particularly more susceptible to oxidation (Dean *et al*., 1985). Oxidation of sulfhydryl groups or methionine residues of proteins cause conformational changes, protein unfolding, and degradation (Dean *et al*., 1985; Davies, 1978).