Avinashilingam Institute for Home Science and Higher Education for Women

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Monitoring of insecticide in *Momordica charantia* by using QuEChERS Method combined with Liquid Chromatography- Mass Spectrometry and Assessment of its interaction with neurological enzymes by molecular docking

By

Gracy.S

21PBC005

II M.Sc. BIOCHEMISTRY

Department of Biochemistry, Biotechnology and Bioinformatics

A thesis Submitted to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore -641 043.

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

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Certificate

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Signature of the Supervisor

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Signature of Head of the Department

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INTRODUCTION

Bitter gourd (*Momordica charantia*), also known as bitter apple, bitter melon, or balsam pear, is a tropical vine in the Cucurbitales order, Cucubitaceae family, and genus *Momordica*. The plant is widely farmed as a medicinal and vegetable crop in India, China, andSouth East Asia (Behera *et al.,* 2008). Even though the entire plant is edible in nature, bitter gourd is mostly produced for its fruit. Fruits, flowers, and young shoots are utilised to taste a variety of Asian meals. For the little bitter taste, fruits are cooked with other vegetables, particularly in soups. Fruits are utilised in Indian cuisines after blanching, par boiling, or soaking in salt water to remove bitterness (Saeed *et al.,* 2018). *Momordicin* is the functional components of bitter gourd. Momordicin was found at fruit, leaves, roots. In addition, there is a study on the improvement of the functionality, such as obesity, gourd, colon cancer prevention in progress (D. Kwatra *et al*., 2013). In spite of modern medical science and the development of diabetes, hypertension, heart disease, and the occurrence of cancer increases steadily. Bitter gourd has been known for its hypoglycemic effects. The methanolic extract of
M. charantia fruit has ability to increases healing of gastric ulcer (S. Alam *et al.,* 2009). This extract also identified contributes to prevents development of gastric ulcers and duodenal ulcers in rats. Unripe fruits of bitter gourd have been found to have blood sugar lowering capacity, similar to that of insulin and can be used to treat patients with diabetes.

It can be grown all year round in various seasons. Additionally, it has therapeutic value, particularly for people with diabetes and heart problems. India is the world's second-largest producer of veggies after China. With a yield of 17.7 mt/ha and 9.575 million hectares under cultivation, India accounts for 14% of the world's total vegetable production. In India, 167.1 million tonnes of vegetables were produced in 2014–15, with an average yield of 17.6 tonnes per hectare. However, according to the third advance estimates, the production of vegetables climbed by 3% in 2015–20. Bitter gourd (*Momordica charantia*) is one of the important vegetable crops. It is also well known for its medicinal value. (Singh, 2008). *Momordica charantia* has a number of alleged uses including cancer prevention, treatment of diabetes, fever, HIV and AIDS, and infections. The nutritive value of bitter gourd in 100 g of edible portion are carbohydrate 4.2 g, calcium 20 mg, phosphorus 55 mg, protein 2.1 g and iron 1.8

g. It is also rich in Vitamin A 210 IU and Vitamin C 88 mg, which plays a vital role in human nutrition (Singh and Kirtiraj, 2012). Bitter gourd growers used a variety of brands of different technical grade pesticides for controlling insect-pests and diseases with several doses. Infestation of insect-pests and diseases was primarily seen on mature stage of the crop. The primary source of information on pesticides use was agricultural input retailers. The bitter gourd growers used 40 to 60 pounds of pesticides per acre. The yield of bitter gourd in the tropics irreverely limited by a variety of insect and mite pests. Bitter gourd is mainly affected by the insecticide in India. Vegetable growers mainly depend on synthetic insecticides to protect their crops. However, improper application of these pesticides has a significant impact on both human and environmental health (Amoabeng *et al.,* 2017; Forchibe *et al.,* 2017). The identification of pest in the bitter gourd Fruit Fly (Dacus cucurbitae and Ducus dorsalis), Red pumpkin beetles (Aulacophora foveicollis), Cucumber Moth (Diaphnai indica), (Kamal,*2018*). The use of proper pesticide may do not cause any adverse effects on human health.

Insecticides are chemicals to protect agricultural crops from biological hazards such as insect, weeds, fungi and other pests. In addition to their use in agriculture, pesticides are also used to protect human health from the vectors of tropical diseases, such as mosquitoes. However, pesticides are also potentially toxic to humans. They may induce adverse health effects on reproduction, immune or nervous systems and cancer. Before they can be authorized for use, pesticides should be tested for all possible health effects to assess any risk to humans. Hazardous chemicals according to potential health effects can be classified as carcinogenic (to cause cancer), neurotoxic (to cause brain damage), or teratogenic (to cause damage to foetus). This classification process, called hazard identification, is the first step of risk assessment. An example of hazard identification is the classification of substances according to their carcinogenicity to humans carried out by the International Agency for Research on Cancer (IARC), the specialized cancer agency of World Health Organization (WHO). The same chemical can have different effects at different doses, depending on the quantitiy of a person exposed to. It can also depend on the route by which the exposure occurs for example, ingestion, inhalation or injection (WHO, 2015). Age, gender, socioeconomic status, diet, health status, length of exposure and form, pesticide concentration are significant factors on the influence of a pesticide and on people under the influence of pesticides (Güler and Çobanoğlu, 1997, as cited in Oymen, 2014). Classification of an agent as a carcinogenic hazard is an

important indication that a certain level of exposure, for example from occupation, environment, food, etc., could result in an increased risk of cancer.

Risk assessment for pesticide residues in food, as conducted by the Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Meeting on Pesticide Residues (JMPR), establishes a safe intake level. Acceptable Daily Intakes (ADIs) are used by governments and international risk managers, such as the Codex Alimentarius Commission (CAC), to establish maximum residue limits (MRLs) for pesticides in food. MRLs are enforced by national authorities to ensure that the amount of pesticide that consumers are exposed to in the food and they eat over a lifetime will not cause any adverse health effects.

Natural or synthetically produced chemicals can be used as pesticide. They could fall under any of the pesticide classes. The key classes to which the majority of contemporary and widely used pesticides belong include organochlorines, carbamates, organophosphates, pyrethroids, and neonicitinoids. Because of abuse, pests and insects are becoming resistant to common insecticides. Pesticides have been associated to a number of harmful health consequences, including those on the skin, gastrointestinal tract, nervous system, respiratory system, reproductive system, and endocrine system. (Sanborn *et al.,* 2007, Mnif, *et al.,* 2011, Semchuk *,et al.,* 1982).In general, insecticide exposure occurs through food (such as in fruits and vegetables, or in the tissues of fish and other animals we consume), contaminated water, or air we breathe. The most popular, Agriculture uses insecticides that include organophosphorus. Chlorpyrifos (CP) exposure in the environment and CP consumption from food sources may have an impact on people. From chronic CP exposure, which is marked by dizziness, tachycardia, and paraesthesia, acute oral exposure causes seizure-like motor signs, a coma, and even fatality. According to research by (Ramya *et al.*,2021), people who spray pesticides are more likely to get specific cancers, such as Hodgkin lymphoma and colon cancer as well as lung, brain, prostate, and breast cancers.

Imidacloprid (CAS 138261-41-3) is the most commonly used neonicotinoid insecticide in Sri Lanka. It typically manifests as nausea or vomiting, abdominal pain, drowsiness, headache, or dizziness. Despite having lower toxicity, imidacloprid has been linked to a number of cases of serious side effects, such as neuropsychiatric effects, acute kidney injury from rhabdomyolysis, ischemic and metabolic encephalopathy, ventricular fibrillation, multiorgan failure, and even death (Fuke *et al.*,2014). Acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, and thiamethoxam are the main neonicotinoids found in commercial pesticides. To

stop the invasion of sucking insect pests, plants in India are regularly treated with the systemic neonicotinoid insecticide imidachloprid. Imidacloprid was the insecticide that was used the most frequently overall in the parts of Tamilnadu where gourds are grown. According to (Caron-Beaudoin *et al.*,2016), higher doses have also been associated to haematological and cardiovascular effects in human.

According to (Zhou *et al*., 2014), several published strategies have been employed in this situation to remove pesticides from a sample of fruits and vegetables while preserving good operational flexibility. According to (Radford *et al*.,2013), these techniques include homogeneous liquid-liquid microextraction, liquid-phase microextraction, solid-phase extraction (SPE), stir bar sorptive extraction, and supercritical fluid extraction. Recent investigations have concentrated on the use of simple glassware as a solution, which would provide a Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) strategy to get past the issues with the prior techniques for assessing the presence of insecticides in vegetables. The safety of the environment and food depends on knowing how much insecticide is present in the samples. In 2003, a unique sample preparation technique called Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) was created for pesticide multiresidue analysis.

Recently, this method—which offers a number of advantages over conventional approaches was presented as an alluring substitute methodology for sample preparation. Therefore, the method's flexibility, high effectiveness, and ease of analytes identification are what make it so effective. Typically, insecticides are discovered via conventional analytical methods include capillary electrophoresis, enzyme-linked immune absorbant assays, gas and liquid chromatography, etc. (Sangavi*et al.,* 2015). For the purpose of Insecticides found in the ridge gourd have been studied using liquid chromatography-mass spectrometry (LC-MS). Liquid chromatography-mass spectrometry (LC-MS) is a technology that is frequently used for multi-residue pesticide detection in vegetable samples due to its high sensitivity and selectivity as well as its capacity to screen numerous insecticides from various chemical categories in very complex matrixes in a single run.

Molecular docking has become an increasingly important tool for drug discovery. The molecular docking approach can be used to model the interaction between a small molecule and a protein at the atomic level, which allow us to characterize the behavior of small molecules in the binding site of target proteins as well as to elucidate fundamental biochemical processes (McConkey,2002). The docking process involves two basic steps: prediction of the ligand

conformation as well as its position and orientation within these sites (usually referred to as ***pose***) and assessment of the binding affinity. Organophosphorus and neonicotinoid pesticide exposure through direct contact, ingestion, or inhalation may have a harmful impact on human health; greater residue in food increases the risk. Such exposure may have particularneurotoxic effects depending on the amount, frequency, and exposure route. Excito toxic damage to the central nervous system can cause long-term neurological, behavioural, and cognitive problems even when exposed individuals receive sufficient medical therapy (Kelvin*et al.,* 2019).

As the oral exposure to insecticides (by consumption of Bitter gourds) may influence our enzymes by blocking them, the current study examined the interaction of insecticides (which were discovered by LC-MS) with neurological enzymes in humans. By calculating the docking score and the amino interaction with the insecticide atoms, one may analyse the interactions of insecticides with neurological enzymes quickly and cheaply using molecular docking tools like Autodock and Discovery studio. Therefore, based on the binding energy, we may expect that pesticides are having an impact on the enzymes, which may result in an enzyme deficit that causes a number of neurological diseases.

To achieve the study's goal, following objectives were developed.

Objectives of the study

- To analyse the levels of insecticides in the vegetable by (QuEChERS) method combined with LC-MS
- To understand the toxic effects of pesticides on human neurological enzymes by Insilico method

The study was conducted two phases. In phase 1 the analysis of insecticide residue in *Momordica charantia* by QuEChERS extraction method followed by the Liquid Chromatography-Mass Spectrometry (LC-MS) was carried out. In phase 2 the interaction of insecticides present in the sample with the neurological enzymes were determined by molecular docking

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REVIEW OF LITERATURE

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2.1 INTRODUCTION

Pesticides are widely used in agriculture and contribute significantly to increase crop yields. Pesticide residues on consumable food products may pose a health risk if applied prior to or during the growing process and after harvest. Fumigants are a type of pesticide that can be applied directly to food products after harvest. Changes in the types and amounts of pesticides used in agriculture and post-harvest necessitate residue analysis and health risk characterization. Pesticides increase crop yield significantly, whether used prior to production or as a post-harvest treatment (Chen Chen, *et al.,* 2011). Pesticide residues in foods have long been a source of concern, particularly in fresh fruits and vegetables (Wendie Claeys, *et al*., 2011).

Pesticide residue exposure through the diet is estimated to be up to five times greater than exposure through other routes such as air and drinking water (Wendie , Claeys, *et al.*, 2011, Zorka Knezevic, *et al*.,2008).Pesticides are classified in toa main categories according to their use and chemical structure, such as Insecticides, fungicides, herbicides, rodenticides, fumigants, and insect repellents (Nida Besbelli, *et al.*, 2008). Further insecticides are classified into four groups based on their chemical functions: organophosphates, pyrethroids, carbamates, and organochlorines (Types of Pesticides. 2012).

Organophosphates are man-made chemicals that work by inhibiting the enzyme acetylcholinesterase. Pyrethroids are a synthetic version of the natural pesticide pyrethrin, and they include the pesticides cypermethrin and permethrin, both of which were detected in several studies in the vegetables. Carbamate pesticides also work by interfering with enzymesin the nervous system, Because of their environmental effects and persistence, organochlorineshave been largely phased out of the market.

Pesticides are well-known to be a public health concern, and pesticides used in agriculture should have established residue limits and be monitored on a regular basis. Pesticide surveillance typically focuses on proper use and adherence to Maximum Residue Limits (MRL). Maximum residue levels are determined by applying pesticides in accordance with good agricultural practices in controlled field experiments. To assess food safety, observed pesticide levels on foods must be compared to health safety limits or toxicological endpoint values such as the Acceptable Daily Intake (ADI) and the Acute Reference Dose (ARfD), as well as different food consumption patterns (Wendie L Claeys, *et al*., 2011). ADI and ARfD

are pesticide toxicity measures for both chronic and acute toxicity. Exposure to or consumption of a specific pesticide at levels below the health safety limit is considered "safe," but this concentration may be higher than the established MRL. Pesticides have been linked with a wide variety of health effects, ranging from headaches and nausea to cancer (Chen Chen, *et al*.,2011), (Inigo-Nunez, *et al.*, 2010). Pesticides are classified into inorganic pesticides and organic pesticide by its chemical nature. The synthetic pesticide is classified into organochlorine (OCs), cyclodiene, organophosphate (OPs), carbamate, synthetic pyrethroids, nicotinoid, triazole which are commonly used due to its advantages in the field.

Long term exposure to pesticides is a known health risk and it is increasingly being linked to cancer, neurotoxic effects, reproductive health concerns and endocrine disruption. Depending on their method of action, certain pesticides, such as those determined to be xenoestrogens (organochlorines, organophosphates, and carbamates), will have increased effects based on the developmental stage of individuals when exposure occurs (Inigo-Nunez, et al., 2010). The effects of pesticides in general are felt more readily by certain populations, and for the general population dietary intake is considered to be a major route of exposure (Alexandre Nougadere, *et al.,* 2012).

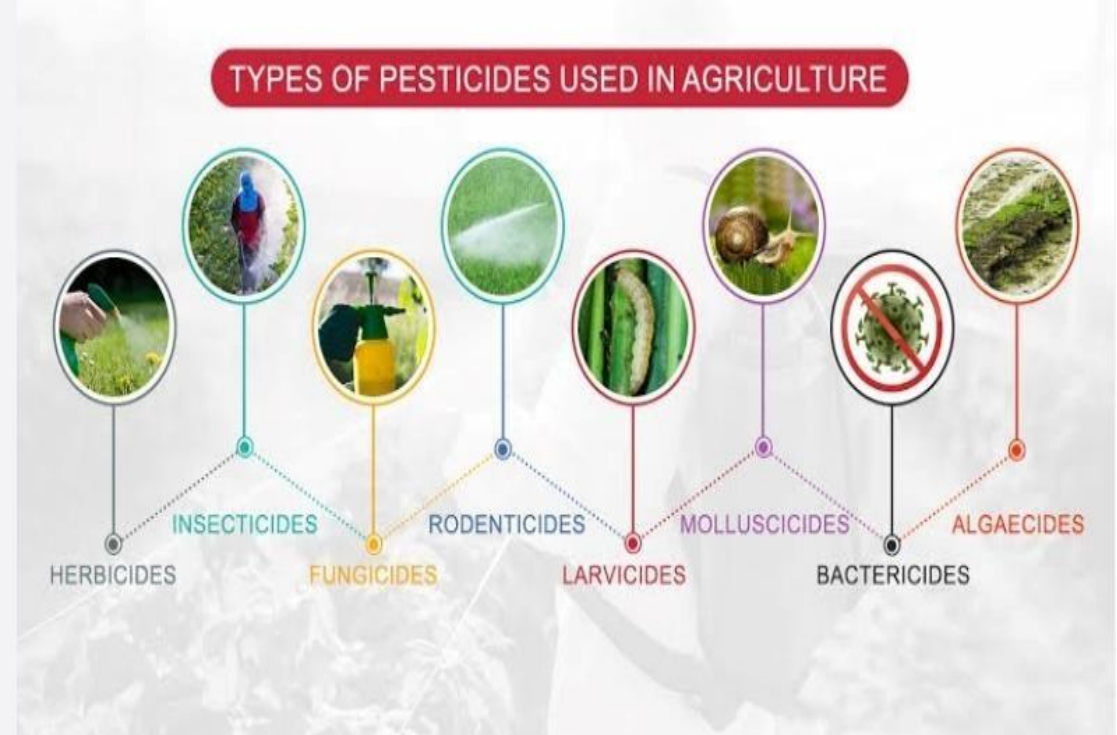


Figure 2.1 Types of Pesticides

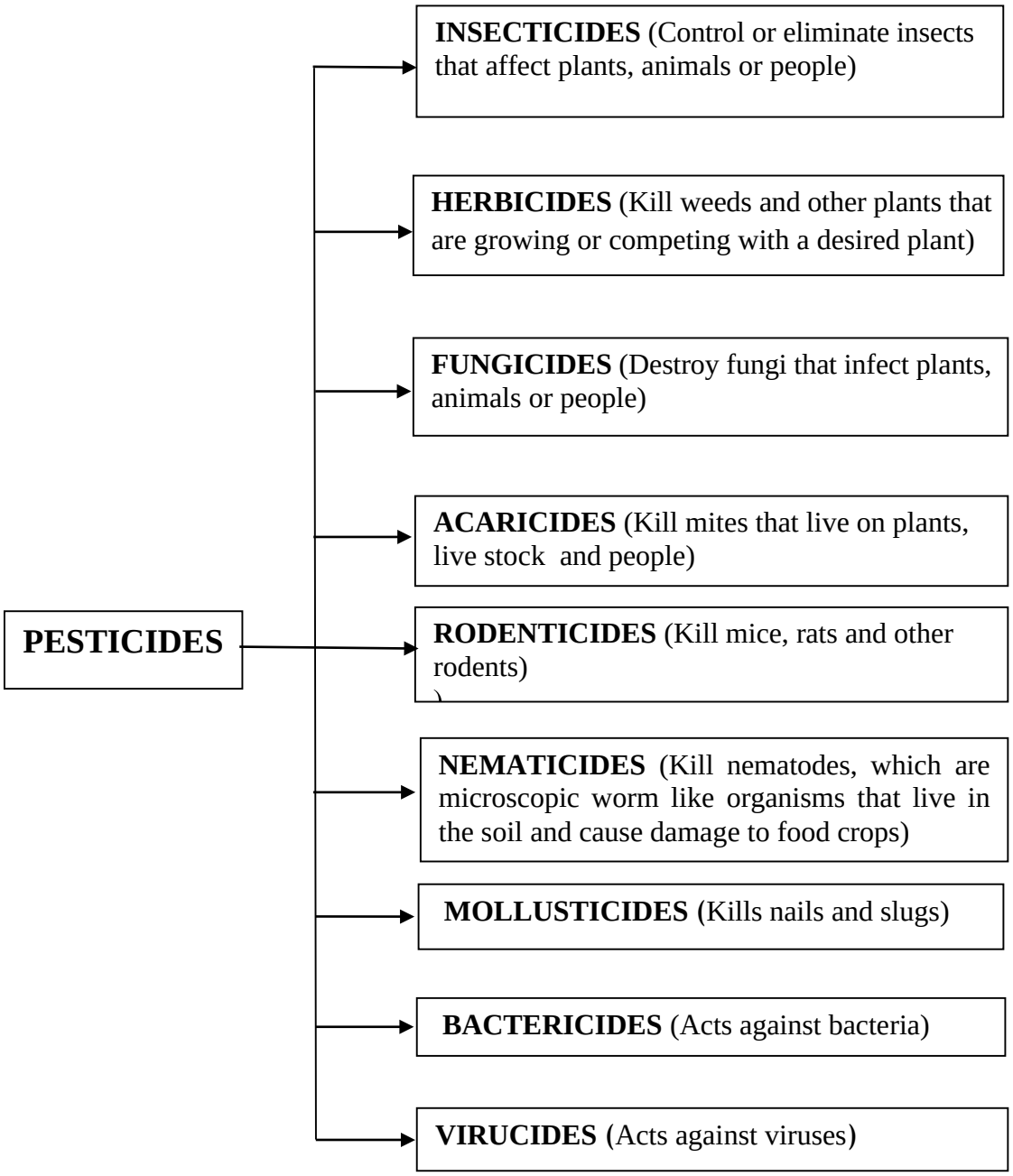


Figure 2.2 Classification of Pesticides

In addition to cooking or deep frying, fruits can be canned, pickled, or dehydrated. It is widely used as a folk medicine to treat diabetes among the indigenous peoples of Asia, South America, India, and East Africa (Joseph & Jini 2013). Aside from the fruits, the roots, leaves, and vines are used to treat toothache, diarrhoea, and furuncles. Bitter gourd products such as bitter gourd tea, also known as gohyah or herbal tea produced from dried bitter gourd slices, are gaining popularity as herbal medicine (Jia *et al*. 2017). Bitter gourd contains an insulin-like

principle known as plant insulin, which has been shown to help reduce blood and urine glucose levels (Janagal *et al*. 2018). Vegetables are any herbaceous plant whose fruit, seeds, roots, tubers, bulbs, leaves, and so on are eaten raw or cooked.

Vegetables have been linked to improved gastrointestinal health, better vision, and a lower risk of heart disease, stroke, chronic diseases including diabetes, and several types of cancer. It is widely acknowledged to be one of the world's most important vegetable crops, with great economic value (Krishnendu *et al.,* 2016). It is an important part of the human diet since it contains carbs, proteins, vitamins, minerals, and other nutrients that are essential for optimal health. It contains a lot of fibre and a lot of vitamins B1, B2, B3, C, magnesium, folic acid, zinc, phosphorus, and manganese (Keding and Krawinkel, 2006). Some of the morphologic properties of *(Momordica charantia)*

Momordica charantia is an annual climbing or trailing herb with stems (vines) up to 5 m long. The stems are ridged, glabrous or hairy, and they bear simple tendrils. The leaves are showy, alternate, simple, borne on 1.5-7 cm long petioles. The leaf-blade can be glabrous or pubescent, deeply palmated, 2.5-10 cm broad x 3-12.5 cm long. The flowers are solitary, unisexual, borne at leaf axils, regular, pentamerous up to 2 cm long, pale yellow to orange-yellow; male and female flowers are distinct. The fruit is a pendulous broadly ovoid and beaked berry, up to 11 cm in length 4 cm in diameter (some cultivars reach up to 45 cm × 9 cm). Immature fruits are green in colour, and then become reddish-orange when ripe. They split open at maturity to release the seeds sheathed in a sticky red pulp. Cultivated fruits have smooth to spiny surface. The seeds are oblong, 10 mm × 5 mm, flattened, white or brown (Njoroge *et al.*, 2004).

The soil condition of the Bitter gourd can be grown on well drained sandy to sandy loam; medium black soils rich in organic matter. Alluvial soil along the river beds is also good for production of bitter gourds. A pH range of 6.0- 7.0 is considered as optimum.

The Climate is a warm season crop grown mainly in sub-tropical and hot-arid regions. They are susceptible to light frost and are provided with partial protection if grown during winter months. Temperature range of 24˚C - 27° C is considered as optimum for the growth of the vines. The seed germinates best when temperatures are higher than 18°C. High humidity at the time of vegetative growth renders the crop susceptible to various fungal diseases.

A research conducted by (Abang *et al.*, 2013) in Cameroon, was revealed that pesticides such as Actellic, Agrizeb, Callomil, Bastion, Beauchamp, Beauchamp, Bravo, Callidim, Callisulfan, Calthio DS, Camindacal, Carbofuran, Cigogne, Cypercal, Cyperdim, Cyperplant, Cyplandim, Cyplandim, Decis, Dimex, Dursban, Furaplant, Gamaline, Gramoxone, Ivory, Karate, Kunter, Lambdacal, Malathane, Nordox, Orthene, Pacha, Parastar, Penncozeb, Planthoate, Plantizeb, Pylory, Pyriforce, Ridomil, Thiodan, Thioplant, Trimangol were found in the tomato, hot pepper, sweet pepper, onion, cabbage, amaranth, okraand eggplant. They observed that weekly sprays of pesticides were the most common practiceamong the participant farmers. And it was sprayed by farmers manually at rate of 40%.

2.2 INSECTICIDE AND TOXICITY LEVEL

Table 2.1 Toxicity Level of Insecticide

WHO CLASS	HAZARDOUS	LD50 for rats (mg/kg of body weight)	
		ORAL	DERMAL
Class- Iα	Extremely Hazardous	Less than 5	Less than 5
Class-Ib	Highly Hazardous	5 to 50	5 to 200
Class-II	Moderately Hazardous	50 to 2000	200 to 2000
Class-III	Slightly Hazardous	Over 2000	Over 2000
Class-V	Unlikely to present acute hazard	5000 or higher	

2.3 INSECTICIDE RESIDUE IN VEGETABLE CROPS

2.3.1 LETTUCE

Lettuce is a leafy vegetable that is commonly used in salads, soups, sandwiches, and wraps, among other things. Though it is a good source of vitamin K and C, as well as folate and iron, contaminated samples were found to be unsafe and risky for human consumption. (Amoah *et al.*, 2006) conducted a study in lettuce contamination in Ghana, discovered that pesticide residue levels exceeded the Maximum Residual Limits in the majority of cases

(MRL). According to the data, 78% of lettuce samples contained chlorpyrifos residues, while only 14% had no detectable pesticide residue. The average value of chlorpyrifos residues obtained was 1.6 mg/kg, which was higher than the standard MRL of 0.5 mg/kg.

In Egypt, Dogheim *et al*., (2004) discovered that lettuce was contaminated with Chlorpyrifos and Profenofos residues, with an average chlorpyrifos residue concentration of 2.8 mg/kg. Bempah *et al.,* (2012) investigated insecticide contamination in a lettuce samples, but only Methoxychlor and Endrin were found to exceed the limit of MRL in the lettuce.

2.3.2 OKRA

Okra is high in fiber, vitamin C, calcium, potassium, and folate (Corleone, 2014). In Uttar Pradesh, (Arora, 2009) tested okra samples and discovered Chlorpyrifos and Cypermethrin residues above the recommended MRL value of 5.75 mg/kg and 0.63 mg/kg, respectively, compared to 0.2 mg/kg (MRL). (Charan *et al*., 2010) discovered 32% contaminated okra with Methyl parathion residues in the Central Aravalli region of Rajasthan, with 4% exceeding the recommended MRL.

Watermelon was the primary crop sprayed with pesticides, but pesticide effects were also observed in the non-target okra crop. Methamedophos, Enthoprophos, Phorate, Diazinon, Dimethoate, Chlorpyrifos, Fenitrothion, Parathion, Profenofos, Malathion, Lindane, Heptachlor, Aldrin, Endosulfan, Dieldrin, DDT, and Endrin were among the pesticides found to be above the MRL in watermelon and okra. The residues of phosate, dimethoate, chlorpyrifos, and malathion were found to contain 19.40 mg/kg, 50.60 mg/kg, 1321.10 mg/kg, and 23.30 mg/kg, respectively, compared to the recommended values of 0.7 mg/kg, 2.0 mg/kg, 10.0 mg/kg, and 3.0 mg/kg respectively. (Mukherjee, 2003) investigated violations in the presence of chlorpyrifos residues during research in Delhi. She discovered that 28.57% of the okra samples exceeded the MRL value (Ranga Rao *et al.,* 2009).

2.3.3 CABBAGE

Cabbage is a leafy vegetable that is high in minerals and vitamins. It is also said to be a good source of Vitamin K and C. It also contains plenty of folate and some forms of Vitamin B. (United States Department of Agriculture Research Service, 2015). Cabbage is used in pickles, salads, and a variety of other dishes. (Bempah *et al.,* 2012) discovered contamination of 68% of the samples with various insecticide in Ghana, as well as residue. Investigated that

the levels of Lindane, Methoxychlor, and Dieldrin were higher than the recommended MRLs, namely 0.100 mg/kg, 0.023 mg/kg, and 0.035 mg/kg, respectively, when compared to the standard value of 0.01 mg/kg.

In Rajasthan, (Charan, *et al.,* 2010) found 28% of the cabbage to be contaminated. With monocrotophos residue and it was 5.12% higher than the MRl value. Smilarly he noticed and chlorpyriphos residue was 2.56% higher than the standard MRL. Osei-Fosu *et al,* (2014) discovered Dimethoate pesticide contamination in vegetables as well as pesticide residue in the samples.

Dimethoate levels were found to be 550-700% higher than the recommended MRL value. (Sapbamrer and Hongsibsong 2014) discovered Chlorpyrifos contamination in 12.5% of Chinese cabbage samples in Ghana. The average pesticide concentration found was 2.86 mg/kg, compared to the recommended level of 0.5 mg/kg. Similarly, (Yu *et al*., 2016) investigated insecticide contamination in Chinese cabbage. where the, the measured level of pesticide residue was found to be which was 3.6% higher than the MRL for Methamidophos, 3.6% higher than the MRL for Dichlorvos, 7.1% higher than the MRL for Omethoate, 10.7% higher than the MRL for Phorate, 3.6% higher than the MRL for Diazinon, and 7.1% higher than the MRL for Parathion.

2.3.4 CUCUMBER

Cucumber is a creeping vine with underground roots. It contains essential nutrients and is high in Vitamin K. It can be used in salads, pickles, and other dishes. A number of studies have revealed the presence of pesticide residues in vegetables. (Bempah *et al*., 2012) discovered Methoxychlor, Dieldrin, DDT, Diazinon, Pirimiphos-methyl, Chlorpyrifos, Profenofos, and Malathion pesticide residues in 43.3% of the total samples in the Ghana region. (Sapbamrer and Hongsibsong 2014) discovered Monocrotophos residues in 75% of Cucumber samples, with an average value of 0.10 mg/kg compared to the standard 0.01 mg/kg (Osei-Fosu *et al*., 2014).

Insecticide Dimethoate, Chlorpyrifos, Malathion, and Fenitrothion are present. For the pesticides tested, the levels were reported to be higher than the MRL such as 25- 550% for Dimethoate, 10- 40% for Chlorpyrifos, 40- 100% for Malathion, and 1200- 1400% for Fenitrothion. Similarly, (Yu *et al.*, 2016) reported Methamidophos, Dichlorvos, Omethoate,

Phorate, Dimethoate, Diazinon, Parathion-methyl, Fenitrothion, Malathion, Fenthion, and Parathion contaminations with levels 3.2% higher than the standard MRL in parathion.

2.3.5 TOMATO

The tomato is a popular vegetable that is eaten in a variety of ways all over the world. It is used in salads, ketchup, soups, and juices, as well as in most vegetable and curry preparations. Tomatoes are high in vitamins, particularly vitamin C. According to Bempah et al. (2012), pesticide residues from Lindane, Methoxychlor, Dieldrin, DDE, DDT, Diazinon, Dimethoate, Pirimiphos-methyl, Chlorpyrifos, Profenofos, and Malathion contaminated 50% of total tomato samples. Similarly, (Szpyrka *et al.,* 2015) stated that 50% of samples in Poland were contaminated with Azoxystrobin Boscalid, Cyprodinil, Dithiocarbamates, Fludioxonil, and Famoxadone. (Ranga Rao *et al.,* 2009), also reported the presence of pesticide residues of Monocrotophos, Chlorpyrifos, Endosulfan, and Cypermethrin in tomato samples in Delhi.

2.4 ROUTES OF EXPOSURE

The three routes of exposure for pesticides are oral ingestion, dermal absorption, and inhalation. Lawn and garden pesticides are used in homes and gardens, on golf courses, along highways and hydro rights-of-way, and in public parks, exposing people by all three routes. Pesticides can be tracked into homes, or brought home from work place on clothing and in vehicles, exposing family members as well. Pesticides are used in pet flea collars, in treatments for scabies and lice, and for home infestations of wasps, cockroaches, and ants. Agricultural pesticides are used on farms, greenhouses, and Orchards, and consumers eating produce and other food products ingest them. Pesticides used domestically or in agriculture run off into ground and surface water, exposing entire populations.

2.5 PRETREATMENT AND EXTRACTION METHOD

A wide range of pre-treatment and extraction techniques are being used for the determination of pesticide residues in fruits and vegetables. The standard methods for the extraction of pesticides in laboratories. The extraction procedure follows a common pathway involving the release of desired analytes from the matrices, followed by a cleanup process which refers to step or series of steps in the analytical procedure in which the bulk of the potential interference co-extracts are removed by physical or chemical methods (solid - liquid or liquid-liquid extraction).

The extraction procedure initially deals with the preparation of sub-samples. The starting material consists of 0.5 to 2 kg samples, which are homogenized by a mixer after cleaning. The homogenized sub-samples ranging from 0.5 to 100 g are taken for extraction. The most commonly used solvents for analyzing pesticide residue in fruits and vegetables are acetonitrile, ethyl acetate, dichloromethane, methanol and toluene. In certain cases, the mixtures of solvents are used to improve the recovery of the methods. In addition, the use of agents such as sodium hydroxide and acetic acid are used to neutralize the matrices and in turn to produce better recovery (Andrade *et al*., 2015). Because of their simplicity, liquid-liquid extraction (LLE) and solid phase extraction (SPE) were used in the extraction process.

However, due to its micro scale extraction process, the use of the QuEChERS method has increased dramatically over the last decade (Lehotay, *et al.,* 2010). Extraction of organic compounds from various matrices (e.g., food, biological, and environmental) is a time-consuming process, but the QuEChERS method shortens the analysis time, reduces the number of analysis steps by using fewer reagents in smaller amounts, and provides high recovery. Over the last few years, the extraction procedure has evolved to simplify the sample preparation process, reduce analysis time, and reduce the use of toxic solvents.

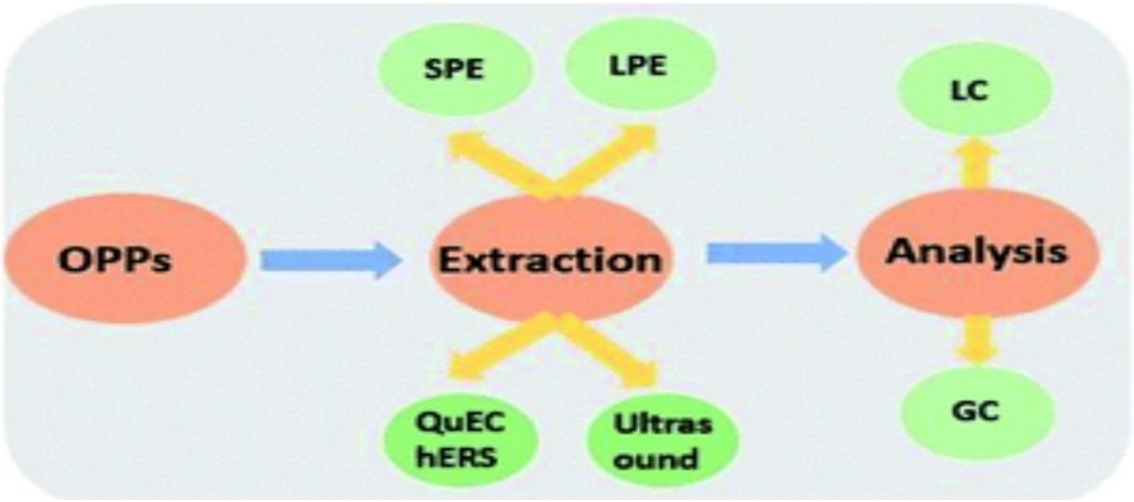


Figure 2.3 Types of Extraction methods in pesticides

2.5.1 LIQUID LIQUID EXTRACTION

Liquid liquid extraction (LLE), also known as solvent extraction and partitioning, is a method of separating compounds based on their relative solubilities in exceptional immiscible liquids. LLE is one of the oldest and most well-established methods for pesticide extraction

because it is dependable, adaptable, and compatible with the majority of instruments. For the LLE, various extraction solvents such as hexane, acetonitrile, and ethyl acetate are used, which is one of the most commonly used medium polarity solvents for pesticide extraction from its matrices (Cho *et al.*, 2013).

In addition to the solvents listed above, (Wang *et al.*, 2012) used the LLE method with a chloroform/dichloromethane mixture to identify seven neonicotinoid insecticides (nitenpyram, dinotefuran, clothianidin, thiamethoxa, acetamiprid, imidacloprid and thiacloprid). The limit of detection (LOD) was obtained in the range of 0.002 - 0.005 mg/kg with a correlation coefficient (R2199) of 0.99, and the method recovered between 76 and 123%. (Grimalt *et al.*,2010) developed another LLE method for the simultaneous detection of eleven pesticides from different classes. Before analysis, the extraction was performed with methanol-water and filtered through nylon syringe filters. Despite the fact that the LLE method is widely used, its major drawbacks include the use of large amounts of toxic solvents, the need for long analysis times, the difficulty of automating it, and its ineffectiveness against polar compounds. Furthermore, the LLE method is not specific to any analyte and extracts all molecules from the matrix, resulting in high matrix interferences.

2.5.2 SOLID PHASE EXTRACTION

Solid phase extraction (SPE) is the most commonly used methods due to its simplicity, rapidity and its ability to treat a large volume of samples with high recovery. For the pre-treatment and determination of pesticide residue in fruits and vegetables, a variety of SPE cartridges are used. A few methods for determining pesticide residues have been reported by florisil columns, C18 columns, and Envi-carb cartridges. (Liu, *et al.*, 2010) developed an extraction method for analysing neonicotinoid pesticides using a SPE HLB cartridge and an Extrelut NT 20 column. (Balinova *et al*., 2007) developed an extraction method that makes use of sorbents with different retention mechanisms (SAX 218 PAX and GCB sorbent).

The majority of residual estimation is done with standard sorbents like primary secondary amine (PSA) and graphitized carbon black (GCB) (Bakirci *et al.*, 2014). Sorbents (PSA-GCB-C18) are sometimes used together in the cleanup process to improve the sensitivity of the method. In addition to the sorbents mentioned above, multi-walled carbon nanotubes (MWCNTs) are used in pesticide extraction due to their effectiveness. (Fan et al., 2014) extracted thirty-six pesticide residues from spinach and cauliflower using MWCNTs as SPE

sorbent. The LODs were found in the range of 0.1 to 5 g/kg, with recovery rates ranging from 57 to 108% at a relative standard deviation (RSD) of less than 12%. The findings indicate that MWCNTs could have better performance with high polar pesticides. Organic solvents, in addition to sorbents, have been used in individual or mixture form for the extraction and elution of pesticide residues, and it is necessary to obtain an appropriate solvent for its intended purpose.

The solvents used are determined by the molecular properties (ionic and non-ionic) of the pesticide to be analysed. Acetonitrile, methanol, ethyl acetate, dichloromethane, acetone, acetic acid, hexane, toluene, petroleum ether, cyclohexane, and diethyl ether have all been used as solvents. The SPE method is said to be the fastest and most efficient method for pesticide analysis. Although SPE methods provide better separation and recovery from complex matrices, mastering their use can be a time-consuming process. Furthermore, this method causes cartridge clogging by the samples suspended matter and has the possibility of low recovery by the interaction of sorbents towards the analytes.

2.5.3 SOLID PHASE MICROEXTRACTION (SPME)

Solid phase microextraction is a simple sample preparation method developed by (Arthur and Pawliszyn, 1990) that is solvent-free, fast, portable, and simple to use. SPME works by dividing analytes between a phase immobilised on SPME fibre and the matrix. (Zhang, *et al.,* 2017) used the SPME method in conjunction with a gas chromatography coupled with micro electron capturing detector (GC-ECD) system to determine eight pyrethroids (bifenthrin, fenpropathrin, cyhalothrin, permethrin, cyfluthrin, flucythrinate, fenvalerate, and deltamethrin). The method's LODs ranged from 0.1 to 0.5 ng/g, with a linearity range of 0.3 to 50 ng/g.

(Abdulra'uf and Tan, 2015) have reported a method using headspace-solid phase microextraction in fruits and vegetables and analyzed it by gas chromatography-mass spectrometry (GC-MS). Similarly, (Kin and Huat, 2010) have determined pesticide residues in strawberry and cucumber samples using the headspace solid-phase micro-extraction method using GC-ECD. Another SPME procedure was conducted by (Saraji *et al.,* 2016) for the analysis of organophosphate pesticides (OPPs) (diazinon, parathion, fenthion, 260 chlorpyrifos). The method was based on the SPME fiber coated with porous carbon nanotubes-

silicon dioxide (CNTs-SiO2) nanohybrids united with gas chromatography- corona discharge ion mobility spectrometer (GC-CD-IMS).

The LODs of the selected pesticides were achieved over the range of 0.005 - 0.020 µg/L. As compared to commercial SPME fibers such as polyacrylate (PA) and polydimethylsiloxane (PDMS), this study showed better extraction efficiency. The main disadvantage of this method is the difficulty in sample carryover, the fragility of the fibre, and its limited lifetime in the extraction process. Furthermore, the process's effectiveness is low due to the low PDMS fibre coating and the interaction in the sorption process between the analytes in the fibre and the analytes in the sample matrix.

2.5.4 MATRIX SOLID PHASE EXTRACTION (MSPD)

Barker *et al.*, 1989 first described this method for extracting solid and semi-solid samples. MSPD combines extraction and cleanup into a single step, making the procedure simple and quick, with less sample loss and solvent consumption. The MSPD method, on the other hand, necessitates a large number of variable optimizations, such as sample amount, dispersant material (type and amount), and elusion composition. (Guan *et al.*, (2011) extracted nine OPPs from eight different fruit and vegetable samples (methamidophos, monocrotophos, mevinphos, methidathion, parathion-methyl, malathion, parathion-ethyl, diazion, ethion). MSPD was combined with rapid resolution liquid chromatography-tandem mass spectrometry (RRLC-MS/MS) in the proposed method. The recovery rates ranged from 71.2 to 102.8%, with RSD ranging from 2.0 to 11.8%. The LODs were discovered to be 0.2 g/kg. (Gilbert-Lopez *et al.*, 2010) also conducted a comparison study for 105 pesticides using the modified QuEChERS and MSPD methods.

SPME extraction was carried out using liquid partitioning with acetonitrile saturated with petroleum ether, followed by MSPD with amino propyl as sorbent material and a florisil cartridge for final clean-up. Liquid-liquid partitioning with acetonitrile was followed by dispersive solid phase extraction and further clean-up with GCB, PSA, and C18 sorbent for the QuEChERS method, with final detection using fast liquid chromatography-electrospray time-of-flight mass spectrometry (LC-TOF/MS). According to the research findings, the optimised QuEChERS method outperformed MSPD in terms of extraction efficiency. The LODs were obtained over the range of 0.2 to 10µg/kg with percentage recovery of 70 to 130 %.

2.5.6 QUICK, EASY, CHEAP, EFFECTIVE, TOUGH, AND SAFE METHOD (QUECHERS)

In 2003, a new strategy based on acetonitrile extraction was introduced, followed by a cleanup using dispersive-solid phase extraction (d-SPE) (Anastassiades *et al.,* 2003). This sample treatment procedure was dubbed QuEChERS. This method gained popularity due to its microscale extraction procedure, which is simpler, takes less time, and uses organic solvent compared to all previous methods. Because of its high recovery, acetonitrile is frequently used in the extraction process.

Despite the fact that acetonitrile is miscible with water, it can be separated by the salt out effect before being cleaned up with d-SPE tubes and MWCNTs (Fernández Moreno *et al.,* 2008).To solve this problem, sodium bicarbonate is suggested (Aysal *et al.,* 2007) to improve analyte recovery. (Carneiro *et al.,* 2013) developed the QuEChERS method, which does not require any cleanup. The proposed method detected analytes successfully with detection and quantification limits of 5 g/kg and 10 g/kg, respectively. The method proved to be simple and provided excellent recovery (70 - 120%) with a 20% RSD. The authors also concluded that the matrix effect was within the analysis's bounds.

(Machado *et al.,* 2017) investigated the QuEChERS method for detecting pesticide residues in globe artichoke leaves and fruits samples. For the analysis of 98 pesticides in globe artichoke, a comparison study was conducted using QuEChERS, MSPD, and dispersive ethyl acetate. According to the findings, the QuEChERS method was effective with the addition of CaCl2 in the clean-up step because of its ability to dehydrate the sample and form insoluble calcium salts with catecholic hydroxyls. Furthermore, the method was detected using GC-MS and LC-MS/MS. LOD ranges for GC-MS and LC-MS/MS were 0.005 - 0.025 mg/kg and 0.003 - 0.015 mg/kg, respectively, with 70 - 120% recovery for both detection techniques.

2.6 INSTRUMENTAL DETECTION FOR EXTRACTION

2.6.1 GAS CHROMATOGRAPHY

Mass detection methods with analyzers such as ion trap (IT) (Tao *et al*., 2009), quadrupole (de Oliveira *et al.,* 2012), triple quadrupole (QqQ) (Wu, 2017), and time of flight mass analyzer (TOF) (Cervera *et al.,* 2012) are also used to improve method sensitivity. Furthermore, selective ion monitoring (SIM) (Lima, *et al.,* 2017) or multiple reaction

monitoring (MRM) (Walorczyk, 2008) are used to reduce matrix interference, where the analyte mass to charge ratio (m/z) is concentrated to achieve a lower limit of detection and quantification with less interference.

(Hkova *et al.,* 2009) described a method for analysing pesticide residues using fast gas chromatography in conjunction with negative chemical ionisation mass spectrometry. Ninety percent of GC chromatographic separations are performed on fused silica 30 mm x 0.2 mm i.d., 0.25m columns with helium or nitrogen as the carrier gas. The use of GC methods has declined over the last decade due to the increased use of polar pesticides (low persistence andhigh toxicity), which have been found to be inappropriate for GC detection methods due to their volatility and poor thermal stability.

2.6.2 LIQUID CHROMATOGRAPHY

In the analysis of pesticide residues, a wide range of liquid chromatography-based techniques have been stated, the majority of which are coupled with ultraviolet (UV), photodiode array (PDA), diode array detector (DAD), and mass (MS) detectors. Octadecyl (C18) is the most commonly used stationary phase for chromatographic separation, and gradient mode has been used to reduce runtime for multi-residue analysis. (Wang *et al.,* 2012) described a multi-residue method for determining seven neonicotinoid insecticides using high-performance liquid chromatography (HPLC) with DAD, with separation achieved using an aglient TC-C18 column.

Similarly, (Al-Rahman *et al.,*2012) used HPLC-DAD analysis to determine the degradation rate of the acaricide fenpyroximate in apple, citrus, and grape. Aside from the methods described above, (Wang, *et al.,* 2014) reported a method for determining trichlorfon monocrotophos by HPLC using molecular imprinted solid-phase extraction. Despite the use of liquid chromatography in conjunction with UV, PDA, and DAD systems, it becomes difficult to provide structural information for the identification of residual pesticide content in fruits and vegetables. Using tandem mass spectrometry (MS/MS), mass detection has been used to overcome these structural interventions as well as to provide structural information from molecular masses and fragmentation patterns.

A number of studies are being conducted using liquid chromatography with mass detection, with different reverse phase columns used as the stationary phase, such as C-8, C-12, and C-18, as well as different organic phases (acetonitrile and methanol) and buffers

(formic acid, ammonium formate, acetic acid, ammonium acetate). In some cases, solvent mixtures (water-acetonitrile and water-methanol) are also used in gradient mode with flow rates ranging from 0.2 to 1.0 mL/min (Christia, *et al.,* 2015; Jallow *et al.,* 2017). Because of their ability to ionise both polar and non-polar analytes, ionisation sources such as electrospray ionisation (ESI) are commonly used in MS detection (Dzuman, *et al.,* 2015). For qualitative and quantitative analysis, mass analyzers such as triple quadrupole (QqQ) (Rong *et al.,* 2017) and Q-Trap (Crnogorac *et al.,* 2008) are used. Aside from these analyzers, (Bakirci, 2012) has reported a multi-residue method for analysing 128 pesticide residues with a single quadrupole. Another study (Guan *et al.,* 2011) reported a method for estimating OPPs in fruits and vegetables using a rapid resolution LC-MS/MS method equipped with QqQ-MS and ESI. Furthermore, (Tian *et al.,* 2016) described a method for simultaneously determining penflufen and one metabolite in vegetables and cereals using a modified QuEChERS method equipped with LC-MS/MS.

2.8 EFFECTS OF PESTICIDE IN METABOLIC PATHWAY:

2.8.1 NEUROTOXICITY

Pesticides including organophosphates, organochlorine and carbamates affect central and peripheral nervous system by their toxic effects. Pesticides shown acute or chronic and long-term or short-term effects on nervous system by the high or low-level exposure during adult, childhood or in utero exposure, and it led to very chronic nervous disorders like Parkinson disease (Keifer, Firestone J 2007).

The insecticide (OP, carbamate, organnochlorine) and fungicides act as neurotoxin and they will affect by modulating the synaptic neurotransmission. It is revealed that OPs have two types of effects, one occurred in minutes and show symptoms like headache, nausea, vomiting, pupillary constriction, dizziness and excessive sweating, tearing, and salivation and in case of severe effects other effects include muscle weakness and twitches, bronchospasm, and changes in heart rate and lead to convulsions and coma. OP exposure led to a disorder called OP-induced delayed polyneuropathy, in which axonal region of neuron is effected badly and unable to produce the neuropathy target esterase enzyme, and also cause overstimulation of postsynaptic cholinergic receptors (Keifer, Mahurin, 1997).

The pesticides are divided in to four groups such as organophosphates, organochlorine, pyrethroids and carbamates. Organophosphorus and organochlorine pesticides are used widely in the world to control insect pest attack on crops (Baig et al., 2009).

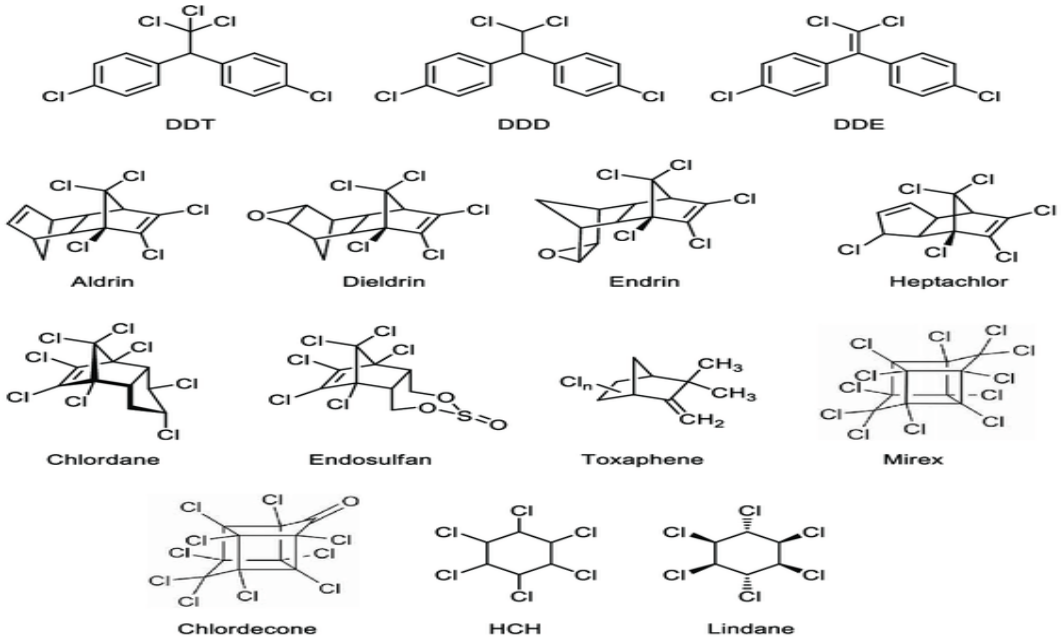


Figure 2.4 Major Pesticides are affected by Neurotoxicity

2.8.2ORGANOPHOSPHATES

Organophosphates (OP) are chemical substances produced by the process of esterification between phosphoric acid and alcohol. Organophosphates can undergo hydrolysis with the liberation of alcohol from the ester bond. These chemicals are the main components of herbicides, pesticides, and insecticides. Acute or chronic exposure to organophosphates can produce varying toxicity levels in humans, animals, plants, and insects. Organophosphates also are widely used in the production of plastics and solvents. Organophosphate pesticides (OPP) are particularly important because of the cholinergicsymptoms produced from exposure. (Robb - 2022, Adeyinka - 2023).

The pesticides are divided in to four groups such as organophosphates, organochlorine, pyrethroids and carbamates. Organophosphorus and organochlorine pesticides are used widely in the world to control insect pest attack on crops (Baig *et al*., 2009).

The most commonly used organophosphate pesticides are the Parathion, Chlorpyrifos, Diazinon, Dichlorvos, Phosmet, Fenitrothion, Tetrachlorvinphos, Azamethiphos, Azinphos Methyl, Malathion, Methyl Parathion. Loewi demonstrated in 1921 that acetylcholine (ACh)is a chemical that can transmit nerve impulses from one nerve to another via synapses. ACh isa neurotransmitter that is derived from acetyl coenzyme A (Acetyl COA). Acetyl COA is

derived from glucose and choline by a reaction catalyzed by choline acetyltransferase (CAT). ACh is stored in the presynaptic membrane in packages called vesicles. Each package is released upon stimulation. Acetylcholine esterase (AChE) uses a hydrolytic process to break down the neurotransmitter ACh into choline and acetate, thereby terminating its effect on the muscarinic and nicotinic receptors. (Rusyniak, Nanagas, 2004).Organophosphates have the ability to irreversibly bind to AChE and prevent the breakdown of ACh. "Liberation" of ACh leads to overstimulation of both the muscarinic and nicotinic receptors. Nicotinic and muscarinic receptors are widely distributed in the body.

Nicotinic Receptors

Nicotinic receptors are of two types: Central and peripheral. Central nicotinic receptors Nn or N2 are located in the central nervous system (CNS). They can also be found in the sympathetic, parasympathetic ganglia of the peripheral nervous system (PNS) and the adrenal medulla. Peripheral nicotinic receptors Nm or NI are located at the level of the neuromuscular junctions

Muscarinic Receptors:

All five subtypes of muscarinic receptors (M1-M5) are present in the CNS. The postganglionic peripheral muscarinic receptors provide parasympathetic innervation to the heart, exocrine glands, and the smooth muscles of the internal organs. Innervation of the sweat gland is via the sympathetic postganglionic fibers. (Kalamida *et al.*,2007, Sellin *et al.*,2008)

Organophosphate pesticides in rural areas where extensive use of herbicides, pesticides, and insecticides are common. Exposure can be via food products such as wheat, flour, and cooking oil. Ant and roach spray might also be a potential source of exposure. Routes of exposure include the following: Inhalation, Direct contact, Ingestion. Adverse effects from exposure to organophosphate pesticides can be classified based on the length of exposure.

- Acute - Occurs within minutes to 24 hours
- Subacute - Occurs between 24 hours and 2 weeks
- Chronic - Exposure beyond weeks to years

The main effect of acute organophosphate exposure is poisoning. Organophosphate pesticides can enter the body through the skin and integumentary system, the respiratory system through

inhalation, or through direct ingestion. Inhalation is the most rapid clinical manifestation of organophosphate pesticides. Chronic OP exposure can have the same effects as acute OP exposure. Memory, speech loss, lack of coordination, and impaired judgement are all hampered by chronic exposure.

Chronic OP exposure can also result in flu-like symptoms such as nausea, vomiting, malaise, and weakness. Chronic exposure has been linked to peripheral polyneuropathy. Exposure to some OPs has been linked to the development of cancer. Malathion, diazinon, tetrachlorvinphos, and parathion are classified as possible carcinogens in a report by the International Agency for Research on Cancer. Exposure to organophosphate pesticides or nerve gas is distinguished by their ability to inhibit the action of AChE, the enzyme responsible for ACh breakdown. Organophosphate pesticides bind to AChE irreversibly in plasma, red blood cells, and synapses in both the PNS and the CNS. The accumulation of ACh causes overstimulation of both nicotinic and muscarinic receptors. (Peter, Cherian.2002, Wadia, *et al.*,1974)

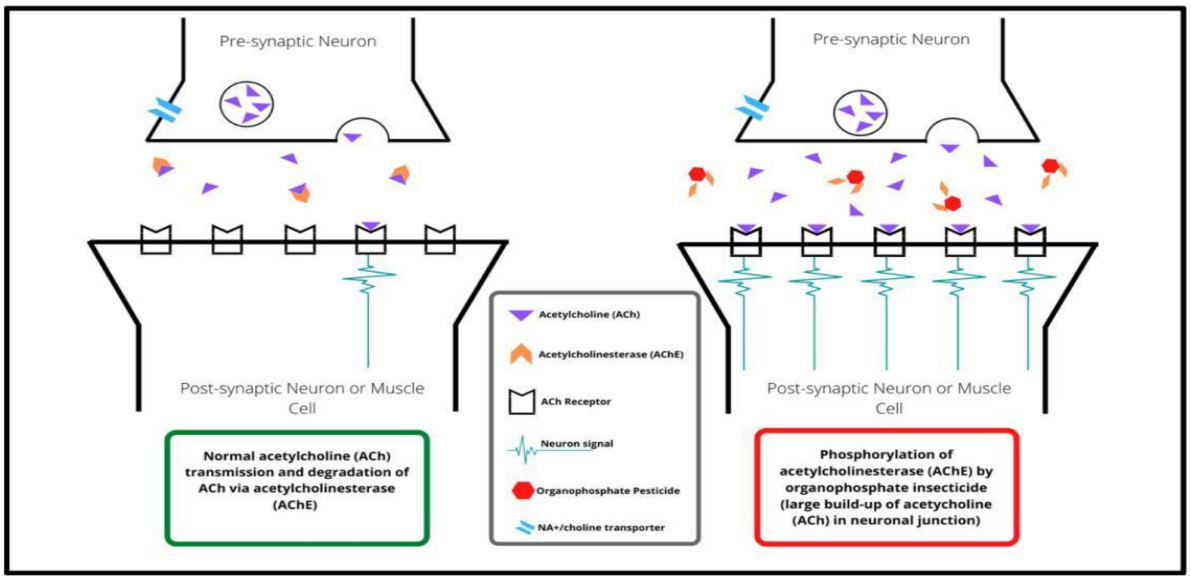


Figure 2.5 Pathway of Organophosphate pesticides

2.7.3ORGANOCHLORINE

Organochlorines (OC) are a group of chlorinated compounds widely used as pesticides. These chemicals belong to the class of persistent organic pollutants (POPs) with high persistence in the environment. OC insecticides were earlier successfully used in control of malaria and typhus, yet they are banned in most of the advanced countries (Aktar *et al.*, 2009)

2.7.4 ALZHEIMER DISEASE

Dementia is decrease in brain capacity, in recent years dementia is increased. One concept about current increased is due to increase in pesticide exposure, may be pesticide increased the dementia pathogenesis. But other research elaborates that pesticide affect neuron function at molecular level by distrusting microtubules and hyperphophorylation which lead to Alzheimer diseases (Zaganas *et al.*,2013).

Organophosphate and organochlorine pesticides are found to effect acetylcholine estrase regulation at synaptic junction in nervous system and may lead to the Alzheimer disease especially in exposed person during their late life (Hayden *et al.*,2010) Another research shows some herbicides (rotenone and paraquat) will disrupt the bio-energetical activites of mitochondria, oxygen metabolism and redox function which lead to Alzheimer disease (Thany *et al.*,2013)

2.7.5 PARKINSON DISEASE

Parkinson disease is generated when dopamine is not produced by the substania nigra neuron (dopaminergic) in brain, which lead to lack of coordination, trembling and loss of muscles control. Research show that some pesticides like rotenone and paraguata will disrupt these dopaminergic neurons and inhibit the production of dopamine and Parkinson disease result (Qi, *et al.*, 2014). It has found that pesticide exposure has some association with Parkinson disease, pesticide and it's metabolites effects mitochondria and modulate xenobiotic metabolism which lead to Parkinson disease (Couteur *et al.*,1999). In separate research it is found that if rats are exposed to the rotenone, then with the passage of time there is neuro-degeneration is found in the peripheral nervous system, there is decrease in motor nerve conduction velocity especially in sciatic nerves. It is due to absence of dopamine and disruption of chemical synapse in peripheral nervous system (Binienda *et al.*,2013)

2.7.6 CHLORPYRIFOS POISONING

Parathion poisoning Parathion pesticide is metabolized into paraoxon (potent cholinesterase inhibitor) in human body by the help of cytochrome p-450 system, and this metabolite is hydrolyzed by paraoxonase (PON), to protect body from toxic effects. It is found that, if PON have Arg/ Arg polymorphism at 192 amino acids then it shows it higher activities in serum. If persons who do not have amino acid polymorphism for high activity of PON gene will be exposed then shows any two or more symptoms like abdominal pain, nausea,

rhinorrhea, dizziness, headache, somnolence, fatigue, gait disturbance, limb numbness, paresthesias, limb pain, or limb weakness, against parathion toxicity (Lee *et al.*,2003). The effected persons have chlorpyrifos metabolism in their urine in the form of 3, 5, 6-trichloro-2-pyridinol. If clinically examined then exposed person unable to discriminate from unexposed person, but symptoms include alteration in nerve conduction velocity, arm or hand tremor, vibrotactile sensitivity, modification in vision and smell sense, or in other words neurobehavioral skills, memory problems, emotional states, fatigue, and loss of muscle strength are also seen in effected persons (Steenland *et al.*,2000)

2.7.7 FETAL GROWTH

It is estimated that 54% women exposed to the pesticides during their pregnancy, they exposed to this pesticide through inhalation, ingestion or contact through skin. In small children exposure is higher and more dangerous because they ingest dust which is contaminated and their breathing zone is closed to ground contain pesticide remains, they spend more time in home with large exposed body surface due to the fewer clothes. And the fetus and children have weak immune system which not able to detoxify pesticide, in case of their exposure may be directly or indirectly so they are more vulnerable (Sabrina, *et al.,* 2010).The estimation of pesticide exposure in fetus is estimated by the analysis of blood from umbilical cord and placenta, but it only shows the recently exposed and persistent pesticides (Rojas et al.,2000)In a different research by examining the samples taking from body parts, hairs, umbilical cord blood and meconium of fetus, it is founded that meconium contain highest exposure to pesticide residue, and it contain almost all potential pesticide to which pregnant female exposed during their gestation period and majority of detected pesticide are used in houses include propoxur, pretilachlor, DDT, cyfluthrin and cypermethrin, blood and hairs not contain all pesticide to which mother exposed during pregnancy so it reviled that meconium is most sensitive part for pesticide exposure in infant (Enrique, *et al.,* 2008).The 5 pesticides (bitertanol, propiconazole, cypermethrin, malathion and terbuthylazine) exposure have high level of endocrine disruption in human. Environmental endocrine disrupting chemicals (EDCs) disrupt endocrine system of fetus if they exposed to them, in utero or early childhood. It causes growth and gestational age defects. Recent research showed that overweight and obesity defect were due to the exposure pesticide extensively used in house and agricultural area and it was lead toward high risk of metabolic and cardiovascular diseases (Wohlfahrt *et al*., 2010) Some persistent pesticideslike organochlorine, polychlorinated biphenyls, and polybrominated biphenyl ethers are

lipophilic and bind with lipids of serum. Other pesticide azole (fungicide) and atrazine effect by increasing gestational length, vrilize female pups and disrupt the endocrine system of fetus, if it was during early phase of gestation then reproductive organ of fetus fail to develop (Rossana *et al*.,2013)

2.7.8 CONGENITAL ANOMALIES

In a study it is found that mother periconceptional pregnancy exposure to pesticide cause various congenital anomalies include orofacial clefts, neural tube defects, conotruncal defects, or limb anomalies, the mothers involve in use of pesticides for house hold gardening or live within 0.25 miles of agriculture agricultural area show high risk of these defects in their offspring (Sabrina *et al.,* 2010). Congenital malformation found attributable fraction of 54.4% (Rojas *et al.,* 2000)

2.7.9 WEIGHT LOSS IN FETUS:

Pesticide exposure effects on growth of fetus especially cause weight loss, exposure to a mixture of pesticides show more adverse effect, in a study on 20 different pesticides (10 insecticides, 6 herbicides, 3 fungicides, and 1 repellant), it is found that 2 pesticide diethyltoluamide and vinclozolin present in greater frequency in blood of umblical cord fetus and fetus weight is inversely proportional to the pesticide number and decreased by a mean of 37.1 g per detected pesticide.

Mixture of pesticide and especially are two fungicides (Vinclozolin and acetochlor) show more harmful effect on fetus growth. Carcinogenic effects of pesticide on fetus Carcinogenic pesticides also affect the fetus during or after gestation, presence of pesticide in maternal cord blood demonstrated that they transfer from mother to fetus during gestation period, and it may increase the risk of cancer. If exposure is before conception, it causes epigenetic alternation in gene expression like imprinting and methylation of DNA in parent's gametes. After conception exposure cause alternation of immunological and hormonal functions and also cause mutation in somatic cell of fetus which cause cancer especially brain cancer (Youn *et al.*,2009)

2.7.10 CHILDHOOD LEUKEMIA

Leukemia is a cancer which causes abnormal production of white blood cells, several studies have showed that childhood leukemia risk increased threefold by the parental exposure of pesticides. According to Children's Cancer Study Group the basic reason of acute non-

lymphoblastic leukemia is parental exposure to pesticides and those children which are regularly exposed to household pesticide have 3.5 times great chance of leukemia (Lawrie, *et al.,* 1997) Pesticide also cause leukemia in children whose mothers are exposed to them during the period of their pregnancy.

This study also indicated that small children less than one year have seven time more chances of leukemia if they are exposed to permethrin pesticide. Another insecticide permethrin used to protects pets from production of fleas and ticks and for killing of mosquitoes, this chemical may alter nervous system working in insects, in some researches it also consider as a carcinogenic. Childhood leukemia occurs due to alteration in the DNA of infants (Ferreira *et al.*,2007)

2.7.11 BLADDER AND COLON CANCER

Aromatic amines used as pesticides are consider as carcinogenic, and produce the bladder cancer in exposed people (Silverman *et al.*,2008) Heterocyclic aromatic amines are found in adduct form in several cases of cancer (Weisburger 2002)

One of heterocyclic aromatic amine imazethapyr is extensively used in agricultural land as herbicides. Research finding show that person who are exposed to that pesticides have 137% increased risk of bladder cancer (Stella, *et al.,* 2009).

2.7.12 THYROID CANCER

Different chemical used to include several pesticides like dioxins, phthalates, polybrominated diphenyl ethers (PBDEs), and other halogenated organochlorines can disturbed the normal thyroid function by the mean of effect hormones production, transportation and their metabolism. Some other chemical which have structural similarities with thyroid hormones and bind with their receptor sites, and destroy the thyroid gland (Patrick, 2009).

In research by Agricultural Health Study (AHS), which is conducted on the incidence of cancer especially thyroid cancer and exposure to a pesticide atrazine. The total 36,357 applicators use atrazine in their field and among them 3,146 are cancer patients and 29 are thyroid cancer patients.

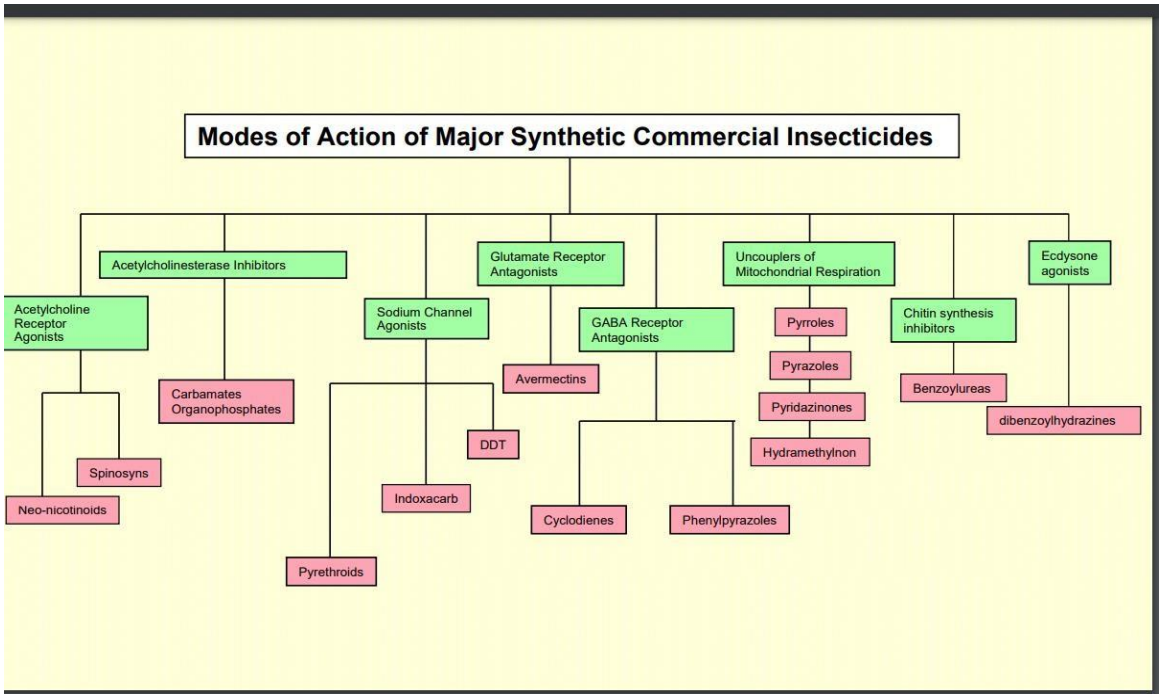


Figure 2.6 Modes of Action of Major Synthetic Commercial Insecticides

2.8 INSILICO STUDIES ON THE INTERACTION OF PESTICIDES WITH METABOLIC ENZYMES

Molecular docking is an important techniques in studying computer-assisted drug designing and structural molecular biology. The major aim of docking protein-ligand is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. This is an efficient technique which helps to understand physiological processes, such as insecticide resistance mechanism, in a range of insects, organisms and systems. Molecular docking is a key tool in structural molecular biology and computer-assisted drug design. The goal of ligand-protein docking is to predict the predominant binding mode(s) of aligand with a protein of known three-dimensional structure. Successful docking methods search high-dimensional spaces effectively and use a scoring function that correctly ranks candidate dockings. Docking can be used to perform virtual screening on large libraries of compounds, rank the results, and propose structural hypotheses of how the ligands inhibit the target, which is invaluable in lead optimization (Verger, 2015)

Docking refers to the process of two molecules are together and forming a stable complex. In the case of enzymes and nerves, docking can occur when the enzyme binds to a receptor on the nerve cell's surface. The docking of a insecticide and enzyme affecting human

nerve could refer to a situation where a insecticide infects a human host and produces an enzyme that interferes with the function of the host's nerves. Insecticide can cause a wide range of diseases in humans, and some of them can affect the nervous system. For example, the parasite responsible for causing malaria, Plasmodium falciparum, can cause cerebral malaria, which is a severe form of the disease that can lead to neurological complications such as seizures, coma, and nerve damage.

Enzymes produced by insecticide can also contribute to the damage of the host's nervous system. For instance, the enzyme phospholipase A2 produced by the parasite Trichinella spiralis can cause inflammation and damage to nerve cells. Docking of enzymes to nerves and the mechanisms by which they affect nerve function is important for developing of the neurological disorders. Therapies that target these enzymes and their interactions with nerves may hold severe conditions such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Salas *et al.,* 2018)

To evaluate insecticide toxicities, humans neurological enzymes is an interesting model. Dichlorvos exerts its toxic effect by irreversibly inhibiting neural acetylcholinesterase. The inhibition provokes the accumulation of acetylcholine in synapses with disruption of nerve function. It also damages the liver, interferes with fatty acid metabolism, and disturbs the antioxidant defence system in rats (Jin *et al.,* 2015). Toxicity ranges from mild skin rashes, eye irritation, vomiting, diarrhoea to severe carcinogenic effects. It causes mutation damaging DNA (Lisi *et al.,* 1987).

Methodology

3

METHODOLOGY

Bitter gourd (*Momordica charantia*), also known as bitter apple, bitter melon, or balsam pear, is a tropical vine in the Cucurbitales order, Cucubitaceae family, and genus Momordica. It contains an abundance of antioxidants, vitamins, minerals, fats, and othernutrients. It is grown in varied environmental settings all over the world, with Turkey, China, India, and the United States producing the most. However, it is frequently attacked by pests, which reduces crop output and quality. Growers rely heavily on synthetic insecticides to protecttheir vegetable crops, because India is a tropical country, insecticides are used at an increased rate (Rajasree *et al.,* 2016).

Insecticides have saved millions of human and animal lives, since their discovery and implementation. They played a critical part in bringing about a revolution in agriculture and human health by controlling agricultural bug pests and vector-borne diseases. More than 80,000 chemical compounds are currently commercially available in agriculture and industry. Approximately 4.6 million tons of pesticides are sprayed to the environment, with insecticides accounting for the majority of overall use in the world to boost food and fibre yield and to reduce the occurrence of vector-borne diseases (Zhang *et al.*, 2011). Despite their value, insecticides have adverse consequences, such as harmful residues in food, water, air, and soil, insect pest rebound and resistance, and effects on non-target organisms. The consequences of insecticides on human health are more dangerous because of direct or indirect exposure; each year, more than 26 million people suffer from pesticide poisoning, with almost 220,000 deaths (Venkatesh *et al.*,2012).

Insecticide residue analysis in vegetables is becoming increasingly relevant. To protect the health of consumers, quality control must be extremely tight. Testing vegetables for residues and pollutants is one of the most essential purposes of food quality assurance. Among chemical risks, insecticide contamination of vegetables has been identified as a key source of many serious diseases as we are intaking the vegetables in our diet on daily basis. Insecticide residues in food may cause cancer, deformities, and damage to the endocrine, neurological, and immunological systems (Abhilash *et al.*, 2009). Several studies have shown that the insecticides are mostly neurotoxic substances they may affect the neurological enzymes like

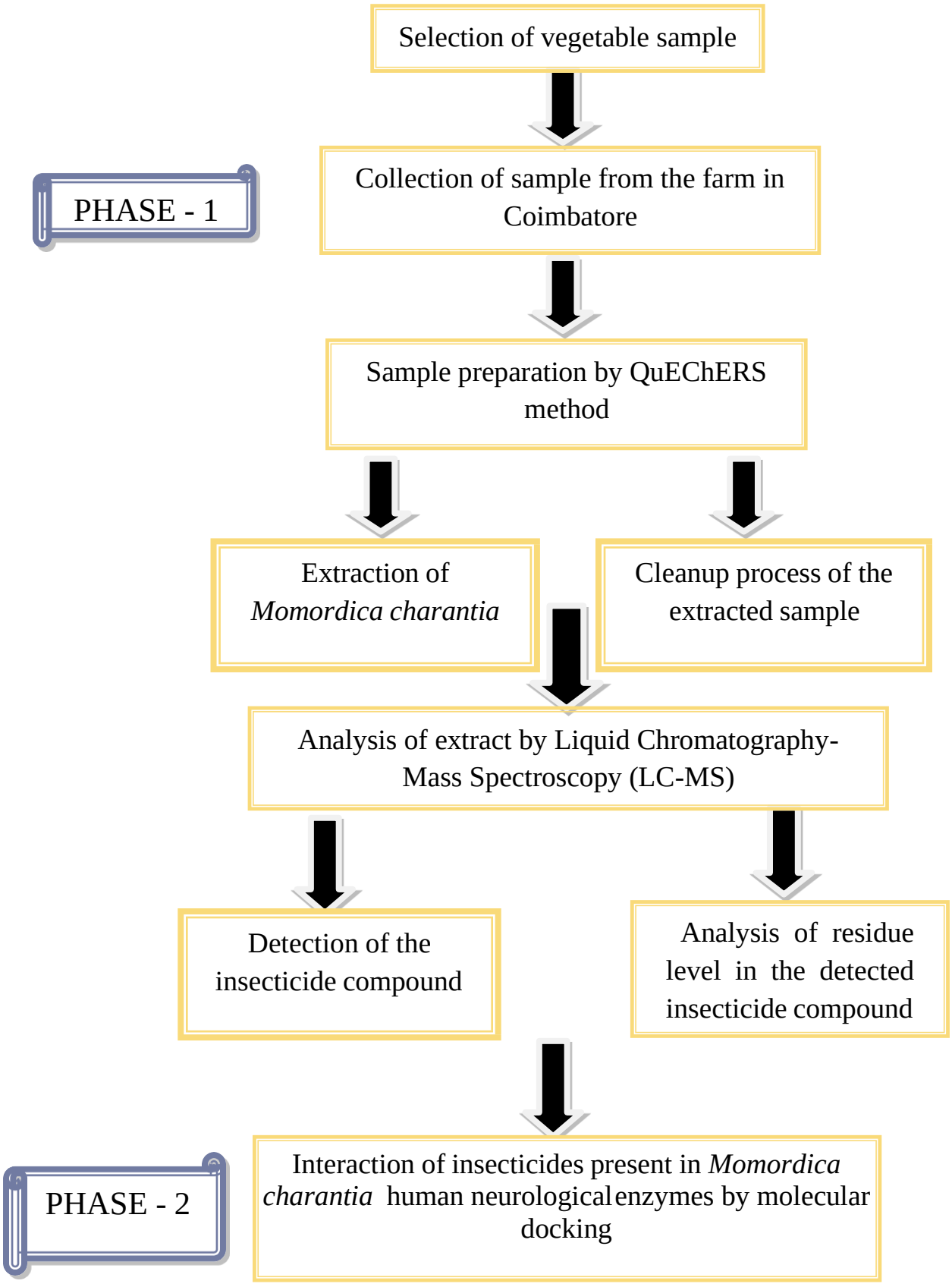
Acetylcholinesterase, Butyrylcholinesterase, Diamine oxidase, etc., which might cause several neurological conditions like Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis (ALS), etc.

To ensure human food safety, the European Community has established maximum residual limits (MRL) for pesticides allowed in animal or vegetable products intended for human consumption. Pesticide residue levels in food and feed were defined in the EU by Regulation(EC) No. 396/2005 of the European Parliament and Council on pesticide residues. To ensure that marketed food commodities comply with food safety legislation, sensitive and depend able analytical methods for determining pesticide residues are necessary.

The "Quick, Easy, Cheap, Effective, Rugged and Safe" (QuEChERS) approach was first announced in 2003 as a quick sample preparation process for pesticide multi-residue analysis. Although the QuEChERS approach is new, it is currently widely 33tilized and has been acknowledged by the international pesticide residue analysis community. The QuEChERS technique begins with an acetonitrile extraction, followed by an extraction/partitioning step after the addition of a salt mixture. The raw extract is subsequently cleaned by dispersive solid-phase extraction (d-SPE). The final acetonitrile extract is immediately accessible to liquid chromatography-mass spectroscopy (LC-MS) for determination (Afify et al., 2010). Multiresidue liquid chromatography-mass spectrometry (LC-MS) technologies are widely acknowledged as an appropriate, highly specific, and exceptionally sensitive tool for analysing food products (Lee *et al.,* 2013).

As the insecticides might affect the neurological enzymes in our body, the interactions can be predicted by molecular docking studies through their binding energy and the structure of enzyme (protein) and insecticide (ligand) interaction can also be identified, for this Autodock and discovery studio has been used in this study.

WORKPLAN:


Selection of vegetable sample
PHASE - 1
Collection of sample from the farm in Coimbatore
Sample preparation by QuEChERS method
Extraction of *Momordica charantia*
Cleanup process of the extracted sample
Analysis of extract by Liquid Chromatography-Mass Spectroscopy (LC-MS)
Detection of the insecticide compound
Analysis of residue level in the detected insecticide compound
PHASE - 2
Interaction of insecticides present in *Momordica charantia* human neurological enzymes by molecular docking

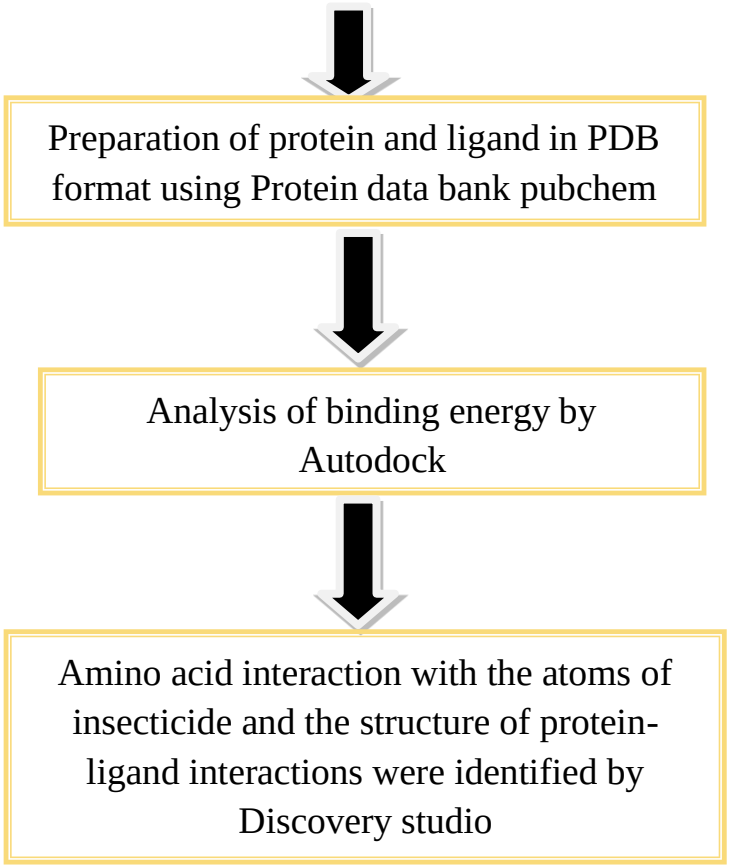


Figure 3.1 Proposed Workplan

The Experimental design adopted in the study "Residual analysis of insecticides in *Momordica charantia* by using QuEChERS method combined with LC-MS and assessmentof its interaction with human neurological enzymes by molecular docking" was conducted in two phases and it is discussed under the following headings:

PHASE-I

3.1 THE ANALYSIS OF INSECTICIDE RESIDUE IN *Momordica charantia* By QuEChERS EXTRACTION METHOD FOLLOWED BY THE LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY (LC-MS)

3.1.1 Sample collection

3.1.2 Extraction and cleanup process - QuEChERS method

3.1.3 Analysis of Extract by Liquid Chromatography-Mass Spectrometry (LC-MS)

PHASE-II

3.2 THE INTERACTION OF INSECTICIDES PRESENT IN *Momordica charantia* THE WITHTHE NEUROLOGICAL ENZYMES BY MOLECULAR DOCKING

3.2.1 Selection of neurological enzymes in humans

3.2.2 Preparation of ligand structures

3.2.3 Preparation of protein structures for docking

3.2.4 Molecular docking

3.2.4.1 Autodock

3.2.4.2 Discovery studio

PHASE-1

3.1 The analysis of insecticide residue in *Momordica charantia* by QuEChERS extraction method followed by the Liquid Chromatography - Mass Spectrometry (LC-MS)

The sample was collected from the local farm, and the extraction of the sample and the clean process was carried out according to the QuEChERS method. Then the extract was given for Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis and then the molecular docking has been carried out in various neurological enzymes along with the result came out from LC-MS analysis by using several bioinformatics tools and websites (Pubchem, PDB, Autodock, Discovery studio).

3.1.1 Sample collection

Bitter gourd (*Momordica charantia*) was the selected vegetable for the study. As perthe farmer's information the ridge gourd has been sprayed with insecticides 3 days before it has been send to the market so according to that the sample has been collected as we need day-1 sample for extraction to get the residue. About 1 kg of sample (ridge gourd) was collected from the local farm located in Theethipalayam near Thondamuthur area in Coimbatore on 4 th February, 2023 (Figure.3). And the sample was stored in the refrigerator at 4°C

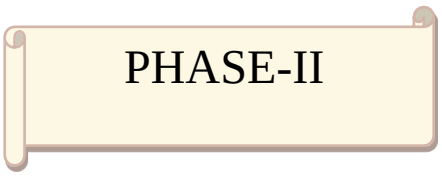
3.1.2 Extraction and cleanup process-QuEChERS method

The extraction and the cleanup procedure was done according to the QuEChERS method. Firstly, the dirt was removed by wiping with dry tissue paper. Approximately 100 g of unwashed fresh samples were chopped by sterilizing knife on the chopping board and then placed into kitchen hand blender. One-inch-thick sample from the skin/upper part of vegetable was taken for analysis. Hence samples include vegetable skin and upper part of the flesh (Figure.4) .10 g of sample was weighed and kept it into the 50ml centrifuge. Here the Acetonitrile was used as the solvent, about 20ml of acetonitrile has been added to the sample kept in the centrifuge tube. Then the salt mixtures (Magnesium sulphate and sodium chloride) were added to it and shaken well by vortexing. After that the centrifugation has been done. Now the cleanup process has been carried out, the supernatant was taken into the prefilled centrifuge tube which has the PSA and magnesium sulphate and GCB, the mixture has been

shaken well by vortexing. Again the centrifugation has been done for the cleanup process. Then the supernatant has been taken and concentrated to near dryness using turbovap. The final volume was then made upto 1ml by adding acetonitrile of HPLC grade (Appendix-1). Then it has been given for LC-MS analysis.

3.1.3 Analysis of Extract by Liquid Chromatography-Mass Spectrometry (LC-MS)

The extracts were analyzed by liquid chromatography-mass spectrometry (LC-MS) technique by the method that was finally selected for the experiment. The detection of insecticide compound and the residue level was made using a single Quadrupole from Shimadzu 2020 series LC-MS (Appendix-2).



3.2 THE INTERACTION OF INSECTICIDES PRESENT IN THE SAMPLE WITH THE NEUROLOGICAL ENZYMES BY MOLECULAR DOCKING

The detected insecticide compounds where then assessed with different neurological enzymes present in our body. The needed protein and ligand PDB format were taken from protein data bank and pubchem website. And the molecular docking has been done by autodock and discovery studio for analyzing the binding energy and the amino acid interaction with the atoms of insecticides and the structure of the protein-ligand interaction were determined.

3.2.1 Selection of neurological enzymes in humans

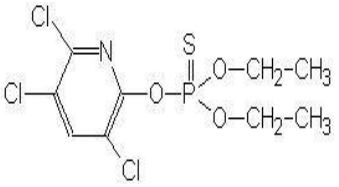
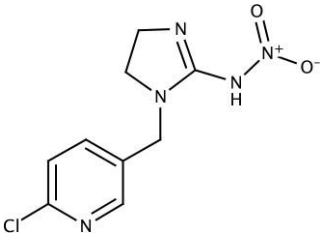
As this study mainly focused on the neurological enzymes in humans, a few of the neurological enzymes were taken for this study to see the ligand (insecticide) interaction. The selected enzymes are Acetylcholinesterase, Glutathione-s-transferase, CytochromeP450, Monoamine oxidase, Nicotinamide mononucleotide adenylyl transferase -S. As the deficiency of these enzymes might causes several neurological conditions.

3.2.2 Preparation of ligand structures

The ligand structures were prepared by using Pubchem. The pubchem is the website of National Center for Biotechnology Information (NCBI). The ligands has been taken as 3D Structure, it was derived as SDF format, as we need only PDB format for docking, the SDF

format was then converted into PDB format using open babel. The data collected from the pubchem were its name, molecular formula, accession number, structure and the molecular weight which has been mentioned in the table.3.1.

Table 3.1 List of Insecticides name, structure, Accession number molecular weight.

S. No	Insecticide	Molecular Formula	Accession No	Structure	Mol Wt g/mol
1.	Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	2730		350.6g/mol
2.	Imidachloprid	$C_9H_{10}ClN_5O_2$	86418		255.66g/mol

3.2.3 Preparation of protein structures for docking

The protein structures were prepared by using RCSB PDB. The RCSB PDB was led by Helen M. Berman, the Research Collaboratory for Structural Bioinformatics **(**RCSB**)** in Brookhaven National Laboratory. The protein structure of the enzymes was derived in 3D. The enzymes were selected according to the Description, species, and Resolution. The speciesfor all the enzymes (Acetylcholinesterase, Glutathione-s-transferase, CytochromeP450, Monoamine oxidase, Nicotinamide mononucleotide adenylyl transferase -S) were *Homo sapiens,* as the study is mainly focused on the human neurological enzymes. The resolution for theenzymes (Acetylcholinesterase-2.12Å, Glutathione-s-transferase-3.30Å, CytochromeP450-2.00Å, Monoamine oxidase -1.60Å, Nicotinamide mononucleotide adenylyltransferase -S-2.30Å). All the protein structures were taken only in the PDB format for docking.The data collected from the RCSB PDB were its name, protein ID, length, chain, and the modelorganism, which was mentioned in the table 3.2.

Table 3.2 The 3D Structure of Proteins Considered For The Studies Are Given Below:

S. No	Protein Name	Protein ID	Length	Chain	Model Organism for study
1.	Acetylcholinesterase	3L11	115	A	*Homo sapiens*
2.	Glutathione-s-transferase	4GTU	217	A, B, C, D	*Homo sapiens*
3.	CytochromeP450	3NXU	485	A, B	*Homo sapiens*
4.	Monoamine oxidase	2V5Z	520	A, B	*Homo sapiens*
5.	Nicotinamide mononucleotide adenylyl transferase -S	1KR2	279	A, B, C, D, E, F	*Homo sapiens*

3.2.4 Molecular docking

The molecular was done by the Autodock 4.2.8 and BIOVIA Discovery studio v3.0. These tools are the most commonly used docking tools and the has the free accession. The autodock has been done for getting the binding energy, whereas the discovery studio has been done for the structures of protein and ligand interaction and the amino acid interactions as well.

3.2.4.1 Autodock4.2.8

For detecting the binding energy of the protein and ligand interaction, Autodock 4.2.8 has been used, which is a free docking software. First the PDB formats of the protein and ligand, the autodocking files and configuration text files has been saved in a separate folder for every individual enzyme, then it has been set in the preference of autodock for easy handling. The protein and ligand has been loaded in PDB format then it has been changed into PDBQT file for running autodock. Then the protein and ligand has been adjusted through the grid box and then after all the steps was done the autodock will run and gives the binding energy. The binding energy was note.

3.2.4.2. Discovery studio

After autodock has been done for collecting the binding energy, the output pdb file which is a complex protein and ligand interaction file, which is needed for deriving the amino acid interaction with the atoms of the insecticides which shows the active pocket site of the protein by where the detected insecticides has been attached for inhibition. And also the protein and ligand interaction structures were also derived from this discovery studio.

Results and Discussion

4

RESULTS AND DISCUSSION

Insecticides are chemicals used to control insects by killing them or preventing them from engaging in behaviors deemed undesirable or destructive. Pesticides are playing important role in agriculture and public health. They make an important role by increasing the production of food and fiber and improving human health by reducing the rate of vector-borne diseases (Blindauer *et al.,* 1999) Insecticides are two types; the first is synthetic insecticides assigned to groups based on the mode of toxic action, such as groups of organochlorines, organophosphates, carbamates, and pyrethroids insecticides; the second is natural insecticides such as azadirachtin, rotenone, spinosad, and abamectin. The extensive and long-term application of synthetic insecticides has been resulted in accumulating their residues in food, milk, water, soil, and other environmental components. It causes adverse health effects to human and ecosystems. Previous studies showed that synthetic insecticides such as malathion, methomyl, chlorpyrifos, pirimiphos - methyl, dimethoate, and *β*-cyfluthrin caused oxidative stress and liver and kidney damage in experimental animals (Akhgari *et al.*,2003). It caused biochemical and hormonal alteration in sprayers of cotton fields (Abbassy *et al.*,2014).

Insecticides have adverse toxic effects in experimental animals. It can induce alterations in biochemical, hormonal, reproductive metabolism, and oxidative stress. It causes also cytotoxic, autogenetic, genotoxic, and carcinogenic effects in experimental animals (Mossa *et al.*,2017)

The current study aims at a comprehensive investigation of the pesticide residual activity of the *(Momordica charantia)* by the LC-MS. In this context, the present study focuses to assess the pesticide residue analysis of *Momordica charantia* and interaction of insecticide with neurological enzymes by auto docking.

The results of the present study were discussed under the following headings:

PHASE -1

4.1 THE ANALYSIS OF INSECTICIDE RESIDUE IN *Momordica charantia* By QuEChERS EXTRACTION METHOD FOLLOWED BY THE LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY (LC-MS)

4.1.1 Sample collection

4.1.2 Extraction and cleanup process - QuEChERS method

4.1.3 Analysis of Extract by Liquid Chromatography-Mass Spectrometry (LC-MS)

PHASE-II

4.2 THE INTERACTION OF INSECTICIDES PRESENT IN THE SAMPLE WITH THE NEUROLOGICAL ENZYMES BY MOLECULAR DOCKING

4.2.1 Selection of neurological enzymes in humans

4.2.2 Preparation of ligand structures

4.2.3 Preparation of protein structures for docking

4.2.4 Molecular docking

4.2.4.1 Autodock

4.2.4.2 Discovery studio

4.1 THE ANALYSIS OF INSECTICIDE RESIDUE IN *Momordica charantia* By QuEChERS EXTRACTION METHOD FOLLOWED BY THE LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY (LC-MS)

4.1.1 Sample collection

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. The basic operation included steps, such as pre-washing,

drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. The pesticide residues were extracted and cleaned up from bitter gourd fruit, by modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method (Anastassiades *et al*., 2003). Ten grams sample was taken in 50 ml polypropylene centrifuge tube and along with 20 ml acetonitrile and vortexed for one min. Then four grams of anhydrous magnesium sulphate (MgSO4) and one gram of sodium chloride were added, vortexed and centrifuged for 10 min. at 6000 rpm. After centrifugation, any remaining moisture was removed by passing the top acetonitrile layer (10 ml) through anhydrous sodium sulphate (4 g). A 6 ml of supernatant was transferred into a 15 ml centrifuge tube containing primary and secondary amine (150 mg), graphitized carbon black (25 mg) and MgSO4 (900 mg), vortexed phase was carefully pipetted into clean glass tube, and was gently evaporated using a turbovap LV (350C) with the stream of nitrogen until nearly dryness.

EXTRACTION OF *Momordica charantia by* QuEChERS method

Plate 4.1 Homogenized sample

The pesticides having the highest health risk in vegetables were Bifenthrin and Difenoconazole as their health risk index was found to be exceeding the cut off value of 1. Imidacloprid was found in all vegetable samples but had no associated health risk, as all the health risk indices for imidacloprid were below the cut off value of 1. By using the QuEChERS method pesticide Glyphosate detected in only one sample had no health risk associated with it. Health risks in fruits were the highest for Amamectin, Bifenthrin, and Difenoconazole and were crossing the

threshold limit of 1. The results reveal the health risk indices of Bifenthrin and Difenoconazole range from 7.8 to 12.46 in vegetables, hence, posing a serious threat to human health. Amamectin, although detected in only fruit and water samples, had the highest health risk of 30.454. Imidacloprid and Glyphosate found in every fruit sample had no associated health risk. The results revealed the presence of pesticides in water, soil, fruit, and vegetable samples. Consumers utilizing these vegetables and fruits are under potential health risks due to the presence of pesticides in soil and water.

The study, an analytical method for detecting pesticides in a variety of environmental matrices including fruits, vegetables, water, and the soil is presented and the health risk associated with the presence of pesticides in a wide range of fruits and vegetables is assessed. It is highly significant because in developing countries agricultural activities contribute majorly toward the total gross domestic product and pesticides are extensively used to control, prevent, devastate, and diminish any harmful pest that destroys crops.

4.1.2 Extraction and cleanup process - QuEChERS method

The samples were extracted by the QuEChERS method and it was processed by clean up method and analysed by LC-MS. So, the below figure shows the before and after clean up the sample. This study describes the combination of two parallel methods in the qualitative and quantitative screening of pesticide residues: (1) qualitative screening for target pesticides by LC-MS/MS using MRM data and (2) confirmation, quantitative determination of the frequently used /or previously detected pesticides using the MRM method. Compared with other available methods, the QuEChERS method is believe to give the best result. This concept was believed to give the widest scope with the least effort and still give excellent qualitative and quantitative results, particularly when using QuEChERS for sample preparation.

Technical developments always follow the way from the primitive via the complicated to the simple. The most common techniques in modern multi-residue target pesticide analysis are gas chromatography, liquid chromatography coupled to mass spectrometry (GC-MS, LC-MS) and/or tandem mass spectrometry (GC-MS/MS, LC-MS/MS) with triple quadrupole mass analysers.

The numerous methods available for pesticide analysis show the importance of this application and rapid pace of developments in analytical chemistry. For example, (Agu" era *et al.*,2000) described a method (Splitless large-volume GC-MS injection for the analysis of

organo- phosphorus and organochlorine pesticides in vegetables using a miniaturised ethyl acetate extraction) for the measurement of only ten organophosphorus and organochlorine pesticides by GC-MS, but over the past decade, the number of pesticides typically included in methods has increased dramatically. The sample preparation techniques have also advanced to complement the analytical techniques depending on the types of analytes and matrices monitored.

Plate 4.2 Before and After Clean up sample of *Momordica charantia*

4.1.3 Analysis of Extract by Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) or High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) is an analytical technique that coupled high resolution chromatographic separation with sensitive and specific mass spectrum detection. Combination of LC with MS is an important development in the history of chromatography (1980s). Mass spectrometry in LC-MS helps to determine the elemental composition and structural elucidation of a sample (Pitt, 2005).

List of Pesticides Screened in *Momordica charantia*:

Table 4.1 Sample Details: Bitter Gourd

Organochlorines (OC)	Residue (mg/kg)	Synthetic Pyrethroids (SP)	Residue (mg//kg)	Organo Phosphates (OP)	Residue (mg/kg)
α-HCH	BLQ(<0.01)	Bifenthrin	BLQ(<0.1)	Phorate	BLQ(<0.05)
β-HCH	BLQ(<0.01)	Fenpropathrin	BLQ(<0.1)	Dimethoate	BLQ(<0.05)

γ-HCH	BLQ(<0.01)	λ-Cyhalothrin	BLQ(<0.1)	Malathion	BLQ(<0.05)
δ-HCH	BLQ(<0.01)	β-Cyfluthrin	BLQ(<0.1)	Chlorpyrifos	2.93
Dicofol	BLQ(<0.01)	α-Cypermethrin	BLQ(<0.1)	Quinalphos	BLQ(<0.05)
Endosulfan-α	BLQ(<0.01)	Fenvalerate	BLQ(<0.1)	Profenophos	BLQ(<0.05)
Endosulfan-β	BLQ(<0.01)	Fluvalinate	BLQ(<0.1)	Ethion	BLQ(<0.05)
Endosulfansulfate	BLQ(<0.01)	Deltamethrin	BLQ(<0.1)	Acephate	BLQ(<0.05)
p,p'-DDD	BLQ(<0.01)			Monocrotophos	BLQ(<0.05)
p,p'-DDT	BLQ(<0.01)			**Fungicide**	
p,p'-DDE	BLQ(<0.01)			Hexaconazole	BLQ(<0.1)
Newer Pesticide Compounds (NC)	**Residue (mg/kg)**	**Newer Pesticide Compounds (NC)**	**Residue (ppb)**	**Newer Pesticide Compounds (NC)**	**Residue (mg/kg)**
Acetamiprid	BLQ(<0.01)	Spirotetramet	BLQ(<0.01)	Novaluron	BLQ(<0.01)
Imidacloprid	4.96	Fluopyram	BLQ(<0.01)	Azadirachtin	BLQ(<0.01)
Thiamethoxam	BLQ(<0.01)	Tetraniliprole	BLQ(<0.01)	Tolfenpyrad	BLQ(<0.01)
Clothianidin	BLQ(<0.01)	Fipronil	BLQ(<0.01)	Flonicamid	BLQ(<0.01)
Azoxystrobin	BLQ(<0.01)	Emamectin Benzoate	BLQ(<0.01)	Spiromesifen	BLQ(<0.01)
Chlorantraniliprole	BLQ(<0.01)	Cyantraniliprole	BLQ(<0.01)	Tebuconazole	BLQ(<0.01)
Flubendamide	BLQ(<0.01)	Spinetorum	BLQ(<0.01)		
Thiacloprid	BLQ(<0.01)				

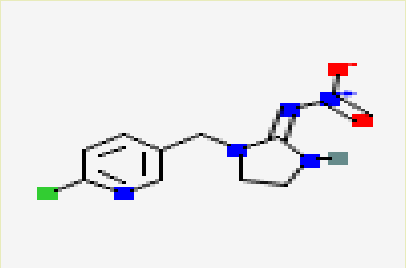
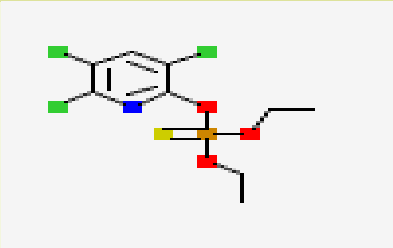
BLQ – Below Level of Quantification

Interpretation	:	The sample analysed was found to contain Imidacloprid (4.96 mg/kg), and Chorpyriphos (2.93 mg/kg) residues.

By using the QuEChERS method they reported that (quick, easy, cheap, effective, rugged, and safe) method of extraction coupled with high-performance liquid chromatographic analysis were carried out, and imidacloprid residues were qualitatively confirmed by liquid chromatography-mass spectrometry. Imidacloprid was not detected in samples of fruit juices and baby foods. It was, however, detected in 38 samples of fruits, vegetables, and cereals, which is about 15.20% of the total samples. Of samples of fruits, 22% showed the presence of imidacloprid, and 2% of samples showed residues above the maximal residue limit. Although imidacloprid was detected in 24% of vegetable samples, only 5.71% showed the presence of

imidacloprid above the maximal residue limit. However, 33% of cereal samples showed the presence of imidacloprid, and about 3% of samples were above the maximal residue limit. The calculated estimated daily intake ranged between 0.004 and 0.131 µg/kg body weight, and the hazard indices ranged from 0.007 to 0.218 for these food commodities. It is therefore indicated that lifetime consumption of vegetables, fruits, fruit juices, baby foods, wheat, rice, and pulses may not pose a health hazard for the population of Lucknow because the hazard indices for imidacloprid residues were below one.

Table 4.2 Results Analysed By LC-MS

NAME OF THE INSECTICIDE	INSECTICIDE RANGE	STRUCTUE OF THE PESTICIDE
IMIDACLOPRID	4.96 mg/kg	
CHLORPYRIPHOS	2.93 mg/kg	

Prodhan et al. (2017) conducted another study in Greece on eggplant. They have detected eleven insecticides (thiamethoxam, cypermethrin, deltamethrin, thiacloprid, acetamiprid, azoxystrobin, chlorpyrifos, dimethoate, propamocarb hydrochloride and chlorpyrifos methyl) in eggplant fruits samples. Among the 142 analyzed samples, 67 (47% of the total number of samples) were found to have pesticide residues and the rest of the samples (53% of the total number of samples) were free from pesticide residues.

The results of this study are in a good agreement with Hasan et al. (2017). They had been detected two types of insecticides (dimethoate and quinalphos) in country bean samples collected from different market places of Dhaka. Among the 50 analyzed samples of country bean, ten samples (20% of the total number of samples) contained residues of dimethoate and quinalphos, of which five were above the maximum residue limits (MRLs). Most of the contaminated samples (8 samples) contained residue of dimethoate.

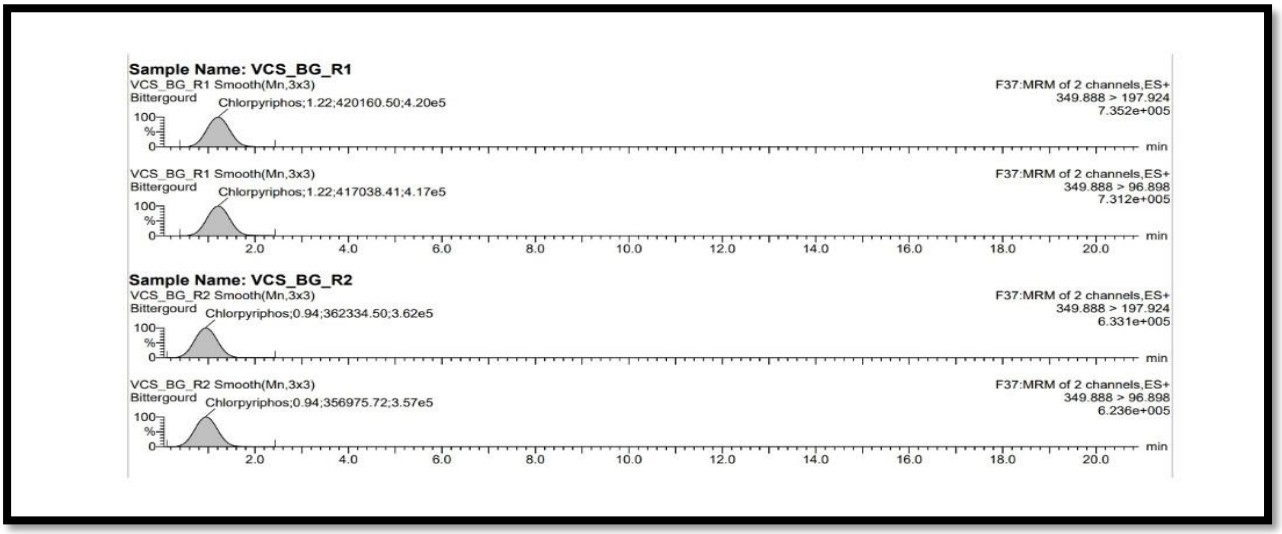


Figure 4.3 Chromatogram of Momordica charantia with chlorpyrifos

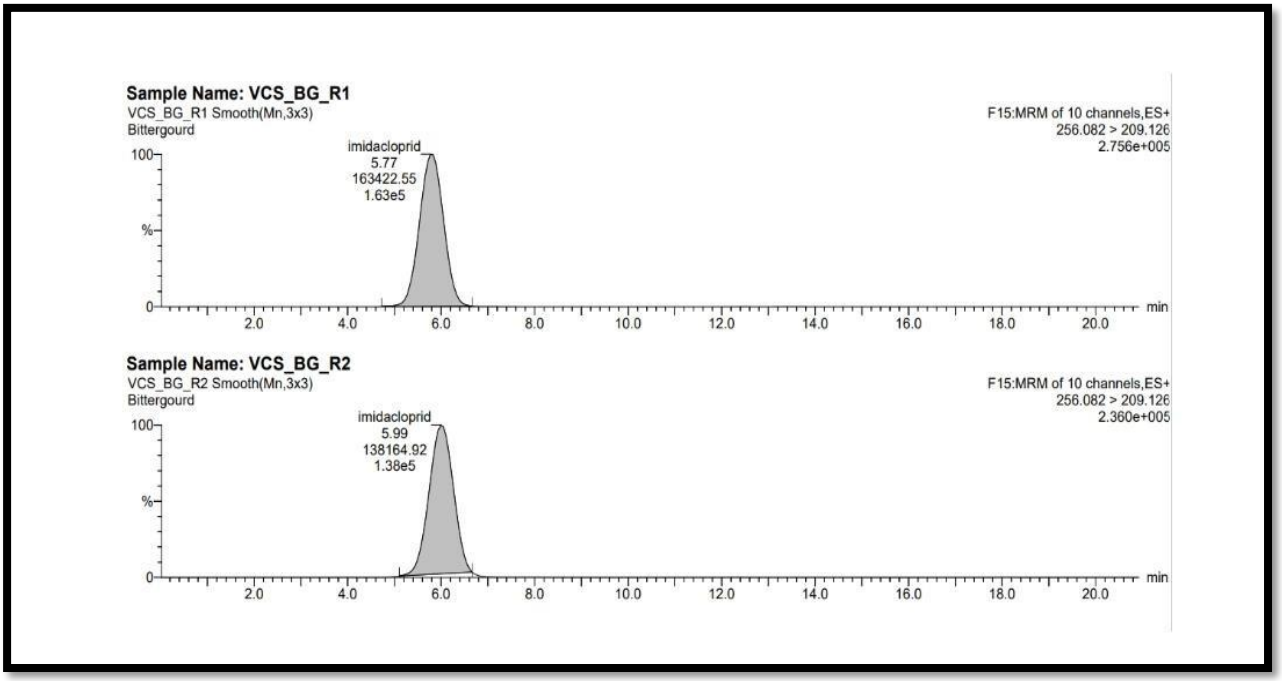


Figure 4.4 ***Chromatogram of Momordica charantia with Imidacloprid***

From pesticide residue analysis of the LC-MS vegetable, it can be concluded that farmers were applying low dose of pesticides in the field during winter season compare to early

summer period. In summer vegetable sample, pesticide residue was detected only vegetable samples. Only organophosphate pesticide Imidacloprid was identified in these sample. The residue concentration was higher than the MRL (0.01 mg/kg). However, lack of awareness and proper knowledge of farmers may create misuse of pesticides which can cause serious problem in vegetable production. This study revealed that pesticide residue in bitter gourd was higher than Maximum Residue Levels.

The concentrations of endosulfans are comparable to those found by Sheikh et al. (2013) in tomato samples from Sindh market in Pakistan, which ranged from nd to 680 ng/g. A study in some fields in Tanzania found higher concentrations of endosulfan of up to 4150 ng/g in tomato samples (Meela, 2009). Another study in fields in Tanzania found p,p'-DDT, dieldrin, β-endosulfan, α-HCH and γ-HCH in tomatoes at concentrations up to 0.62 ng/g (Mtashobya, 2010), which were lower than the concentrations found in this study.

Long term accumulation of pesticides residues in human body via dietary intake of vegetable and other food commodities is an alarming problem. However, the impact of pesticide residues can be minimized by preventive measures such as rational use of pesticides, washing and proper processing of vegetables, practicing organic farming, use of natural pesticides and bio-pesticides, and strict implementation and amendment of pesticide related laws.

The adoption of effective legislation for properly regulating pesticide use and increasing awareness and technical know-how in the farming community should be incorporated. To minimize the pesticide residue level, farmers are suggested to wash the vegetables after harvest from the field, and consumers are advised to wash the vegetables several times with water before cooking.

The findings from the current study showed that the collected sample were contaminated with Imidacloprid and chlorpyrifos residues and the quantified residues were above to their respective maximum residue level (MRL). The results are not in accordance with (Rahman *et al., 2010*) who reported that the diazinin and dimethoate was the frequently detected pesticide from bitter gourd sample. Safique *et al.,* also monitored the pesticide residues from market samples of bitter gourd. It concluded that chlorpyrifos residues were detected at higher level than MRL level.

The results of safique *et al.,* are partly in line with the results of the present investigation. The variations of results from the previous studies were due to temporal variations, different pesticides used by different famous from different locations, dosages used, spraying equipment used and the pest present in the field.

3.2 THE INTERACTION OF INSECTICIDES PRESENT IN THE SAMPLE WITH THE NEUROLOGICAL ENZYMES BY MOLECULAR DOCKING

Protein-ligands docking

The docking results of all the prepared proteins with different insecticides (ligands) are presented in the following tables (4,5,6,7,8). The docking of insecticides to the all the three relevant proteins reveals that at the active pockets of protein series of amino acid residues broadly interacts with all the ligands. A plethora of information was yielded by the complex formed by protein molecules with ligands, highlighted the conclusive role played by numerous factors namely hydrogen bonds, metal interactions, lipophilic interactions and salt bridges interaction in the protein–ligand interaction profile. When the binding of drug molecule to the receptor protein is perfectly done a lead molecule is said to be triggered. The result/output of all the ligands were given by energy values in kcal/mol.

Lowest the energy values strongest is the interaction. To determine all possible determinants of the protein, which would probably network with the ligands, non-covalent interactions like hydrogen bonds were analyzed Maround 5Å distances from the ligand/protein atoms. Such an interaction is comparable to the lock-and- key principle, in which the lock encodes the protein and the key is assembles by the ligand. The major driving force for binding appears to be hydrophobic interaction whose specificity is however controlled by hydrogen bonding interactions.

All the insecticides interacted with all the five proteins. Among all the leads Imidacloprid (Docking score: -5.104) and chlorpyrifos (Docking score:-7.012) for Acetylcholine esterase (Table 4.3); Imidacloprid (Docking score: -6.501) chlorpyrifos (Dockingscore -5.801) for Glutathione-S-Transferase(Table 4.4) and Imidacloprid (Docking score: - 7.734) chlorpyrifos (Docking score: -7.012) for Monoamine oxidase-B (Table 4.5) Imidacloprid(Docking score: -6.843) and chlorpyrifos (Docking score -5.403) for Cytochrome P 450(Table 4.6)Imidacloprid(Docking score: -6.843) and chlorpyrifos (Docking score -5.403)

for Cytochrome P 450(Table 4.7) showed best interaction pose significantly, which revealed thatthese compounds are easily degraded by these detoxifying enzymes in the human system and thus insects are found to be resistant to these insecticides.

Table 4.3 Insecticides interacting with Acetylcholine esterase protein

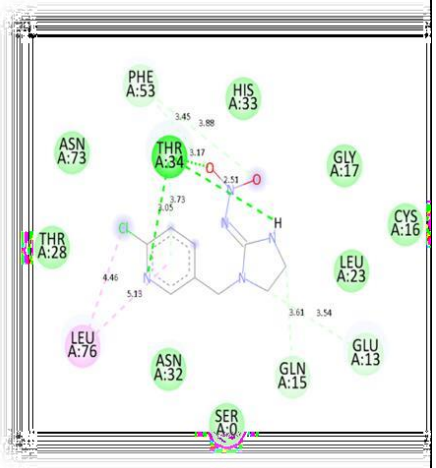
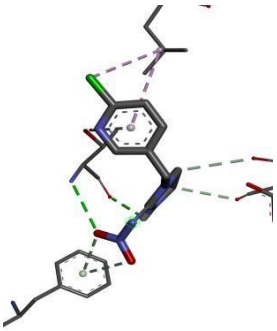
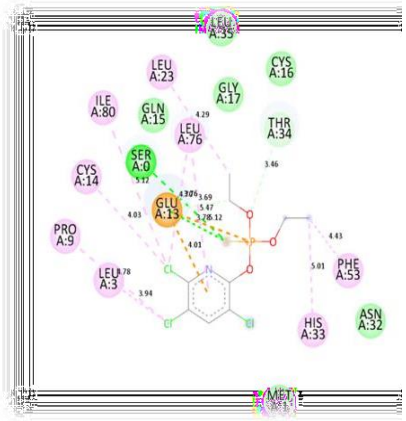
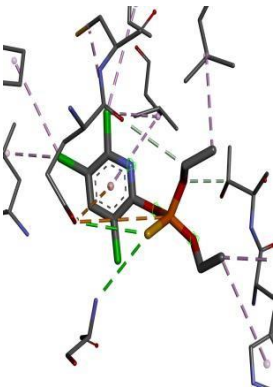
S.No	Insecticides	Docking score Kcal/ mol	Amino acid Interaction with atoms of insecticide	Structure of protein (CE)Ligand interaction
1	IMIDACLOPRID	**-5.104**		
2	CHLORPYRIFOS	**-7.012**		

Table 4.4 Insecticides interacting with Glutathione-S-Transferase protein

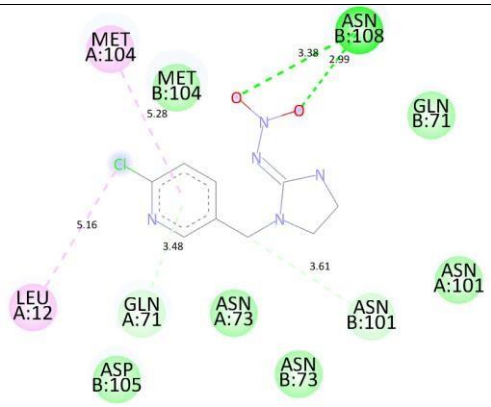
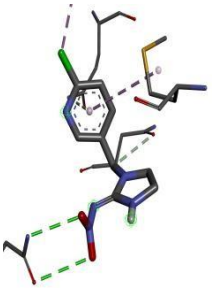
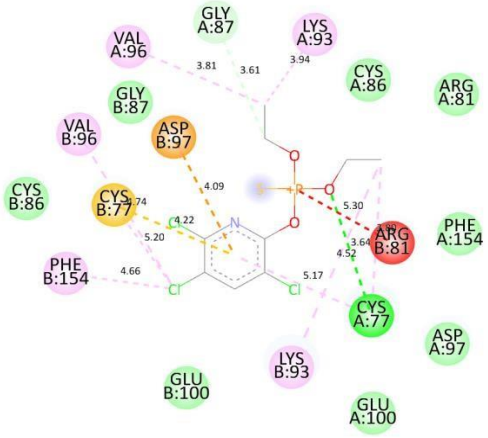
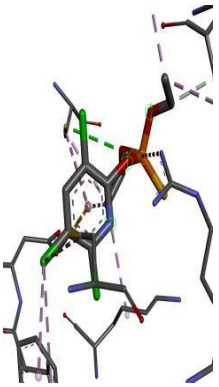
S.No	Insecticides	Docking score Kcal/mol	Amino acid Interaction with atoms of insecticide	Structure of protein (CE)Ligand interaction
1	IMIDACLOPRID	**-6.501**		
2	CHLORPYRIFOS	**-5.801**		

Table 4.5 Insecticides interacting with Monoamine oxidase-B protein

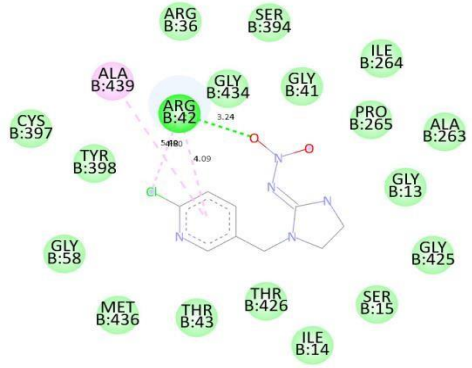
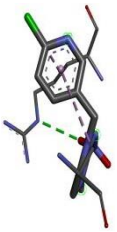
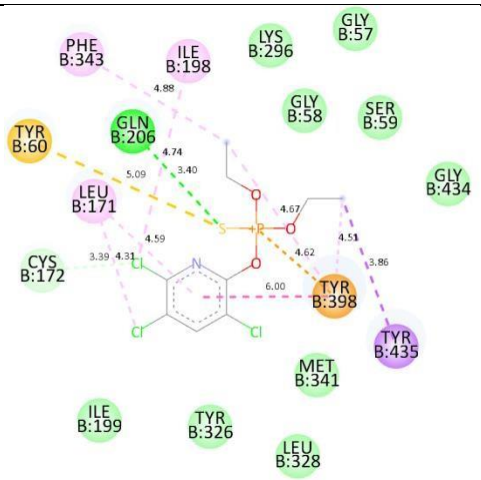
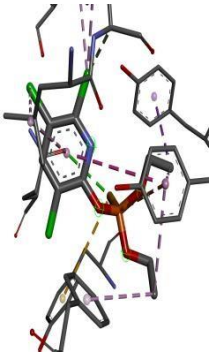
S.No	Insecticides	Docking score Kcal/mol	Amino acid Interaction with atoms of insecticide	Structure of protein (CE)Ligand interaction
1	IMIDACLOPRID	**-7.734**		
2	CHLORPYRIFOS	**-7.012**		

Table 4.6 Insecticides interacting with Cytochrome P 450 protein

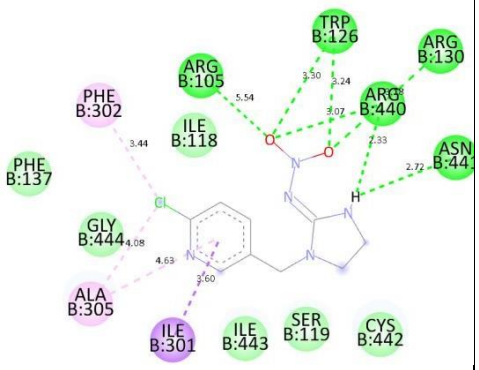
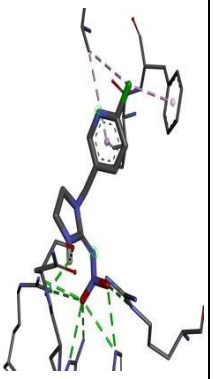
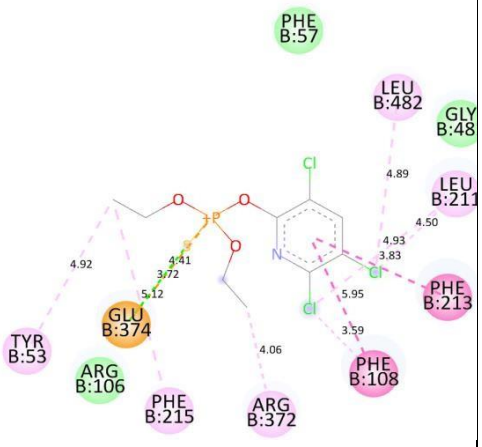
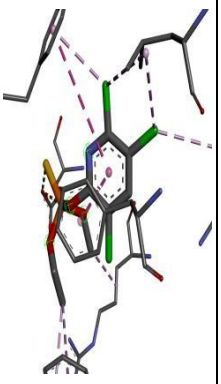
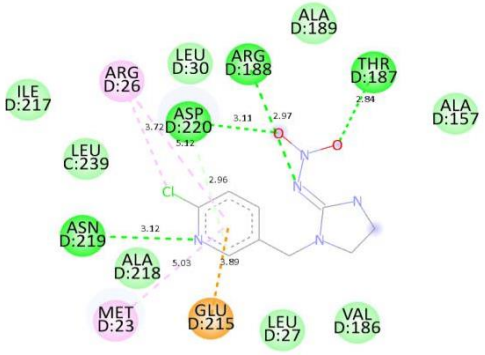
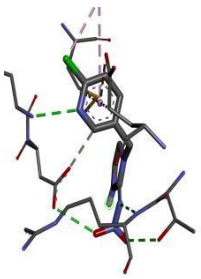
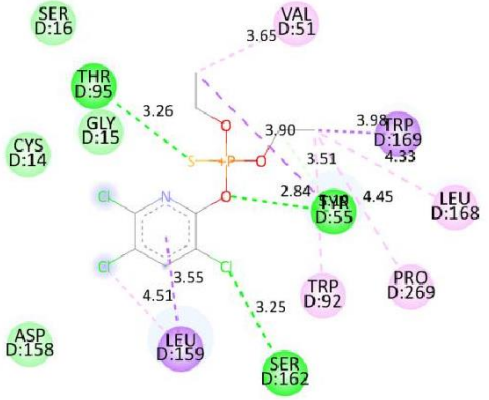
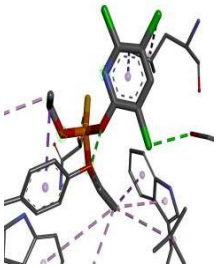
S.No	Insecticides	Docking score Kcal/mol	Amino acid Interaction with atoms of insecticide	Structure of protein (CE) Ligand interaction
1	IMIDACLOPRID	**-6.843**		
2	CHLORPYRIFOS	**-5.403**		

Table 4.7 Insecticides interacting with Nicotinamide mononucleotide adenylyltransferase-S protein

S.No	Insecticides	Docking score Kcal/mol	Amino acid Interaction with atoms of insecticide	Structure of protein (CE)Ligand interaction
1	IMIDACLOPRID	**-7.609**		
2	CHLORPYRIFOS	**-5.707**		

The neural system of the host can potentially be harmed by the enzymes released by pesticide. For instance, the enzyme phospholipase A2 generated by the parasite Trichinella spiralis can cause inflammation and damage to nerve cells. The methods by which enzymes alter neuronal function and how they dock to nerves are crucial for the development of neurological diseases. Therapies that target these enzymes and their interactions with nerves may hold severe conditions such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Salas *et al.*, 2018)

All the two different insecticides interacted with all the five proteins, the specific substrates were used as standard for interaction with the enzymes These docking score after interaction is tabulated in the following tables.

Docking score**: Acetylcholine esterase -** standard specific substrate. Among all the leads(Docking score: -5.104) and chlorpyrifos (Docking score:-7.012)

Docking score: Glutathione-S-Transferase - Standard specific substrate Imidacloprid(Docking score: -6.501) chlorpyrifos (Docking score -5.801)

Docking score: Monoamine oxidase-B - Standard specific substrate Imidacloprid (Docking score: -7.734) chlorpyrifos (Docking score: -7.012

Docking score: Cytochrome P 450 - Standard specific substrate Imidacloprid (Dockingscore: -6.843) and chlorpyrifos (Docking score -5.403)

Docking score: Nicotinamide mononucleotide adenylyl transferas -S - Standardspecific substrate Imidacloprid (Docking score :- 7.609) and chlorpyrifos (Docking score -5.707)

It showed best interaction pose significantly, which revealed that all the insecticides interacting with all the three proteins considered for the studies showed that the insecticides have been degraded by enzymes of human and have developed resistance. Therefore more negative docking score is more, that is proportional to the enzyme activity (human enzyme interaction is more) and corresponding to the efficiency of the insecticide.

A showed stable conformations, with lower energy in the (Two different insecticides-neurological enzymes) ligand-receptor complex of the compounds analyzed in this study, thus having a high affinity towards the active site of the enzymes Acetylcholine esterase**,** Glutathione-S-Transferase, Monoamine oxidase-B, Cytochrome P 450,from a variety of interactions, which can determine its insecticidal potential against the humans neurological enzymes respectively.And here in, the molecular docking of 2 different insecticides against 5 neurological enzymes were studied using Auto dock.

Here each insecticide is corresponding to the Docking score /Binding energy of that particular insecticide with the specific neurological enzyme. Higher docking score more

activation/high interaction with neurological enzyme and insecticide is less efficient, insecticide. Lesser the docking score is less activation /least interaction with neurological enzyme and insecticide is more efficient.

Summary and Conclusion

5

SUMMARY AND CONCLUSION

Pesticides are designed to (in most cases) kill pests. Many pesticides can also pose risks to people. Generally, however, people are likely to be exposed to only very small amounts of a pesticides – too small to pose a risk.

To determine risk, one must consider both the toxicity or hazard of the pesticide and the likelihood of exposure. A low level of exposure to a very toxic pesticide may be no more dangerous than a high level of exposure to a relatively low toxicity pesticide.

The health effects of pesticides depend on the type of pesticide. Some, such as the organophosphates and carbamates, affect the nervous system. Others may irritate the skin or eyes. Some pesticides may be carcinogens. Others may affect the hormone or endocrine system in the body.

The study was conducted in two phases

PHASE-I

THE ANALYSIS OF INSECTICIDE RESIDUE IN *Momordica charantia* By QuEChERS EXTRACTION METHOD FOLLOWED BY THE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

The extraction of the bitter gourd by the QuEChERS method and the extraction was carried out by the LC-MS and the value is above the MRL value it causes adverse effect in humans. Long term accumulation of pesticides residues in human body via dietary intake of vegetable and other food commodities is an alarming problem.

Salient findings

- The sample analysed was found to contain Imidacloprid (4.96 mg/kg), and Chorpyriphos (2.93 mg/kg) residues.
- The high amount of pesticide residue in observed in the Imidacloprid (4.96 mg/kg)

PHASE-II

THE INTERACTION OF INSECTICIDES PRESENT IN THE SAMPLE WITH THE NEUROLOGICAL ENZYMES BY MOLECULAR DOCKING

- The screened ***in-silico*** for, toxicity prediction, and molecular docking against their target actions in the human system.
- Glutathione S- transferases (GSTs) was selected as target protein showing the best-docked score with chlorpyrifos.
- This study reflects revealed various adverse effects on human health and advocated provisions of alternative solutions such as using GST is a binding agents to hold the toxic chemicals out of living system and eventually saves valuable lives of the farmers.

All the insecticides interacted with all the five proteins. Among all the two insecticides, showed least negative value to all three enzymes, which showed least interaction with the detoxification enzymes, thus Imidacloprid, chlorpyrifos are more potential insecticides. All insecticides showed best interaction pose significantly, which revealed that all the insecticides interacting with all the five proteins considered for the studies showed that the insecticides have been degraded by enzymes of human.

Molecular docking is an important tool that predicts the best orientation between two molecules, protein and the insecticide (ligand) molecule. This most effective approach that helps to understand physiological processes such human, in a wide range of organisms and human systems, A showed stable conformations, with lower energy in the different insecticides

neurological enzymes) ligand-receptor complex of the compounds analyzed in this study, thus having a high affinity towards the active site of the enzymes Acetylcholinesterase, and Glutathione S transferase, CytochromeP450, Monoamine oxidase, Nicotinamide mononucleotide adenylyl transferase -S from a variety of interactions, which determines its insecticidal potential against the humans respectively. Therefore, molecular docking is a most efficient technique to understand the molecular interactions, and it is useful to study biochemical processes, since one of the major constraints of agriculture and insect pest control is insecticide, the information from this study will be extremely useful to understand the interaction between the enzyme and insecticide, and determines the insecticidal potential.

Conclusions drawn from the findings of the present investigation

Many pesticide chemicals also lead to different types of cancers in humans. Some farmers use excess amounts of pest repellents, increasing their residues going into the ecosystem and polluting the environment. They cause land degradation and can have negative impacts on soil fertility in the long run.

The study clearly suggested that an extensive usage of pesticide leads to the severe health effects and affect the nervous system. Pesticides misuse can lead to pesticide poisoning, the consequences of which range from mild skin irritations to seizure to death. The effects of poisoning vary drastically depending on dosage and levels of exposure. The residue of the pesticide will remain for 18 days in the vegetable sample. The farmers should cultivate the vegetable sample after 20 days it may don’t affect have adverse effect on human health.

Scope for the future studies

- ✓ A suite of alternatives can potentially reduce pesticide use in some crops, ranging from biologicals/plant-biostimulants, IPM, organic and agroecological practices to technology-driven approaches such as precision agriculture.
- ✓ The outcomes of these research efforts must translate effectively into practice and address ‘on the ground’ challenges. Policy strategies need to provide a robust framework for farmers to take risks in implementing new practices that account for the time needed to implement change.

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Appendices

APPENDICES

APPENDIX-1

QuEChERS METHOD

(Anastassiades *et al.*,2003)

Principle

The QuEChERS method principle is similar to high-performance liquid chromatography (HPLC) and solid phase extraction (SPE). It uses the adsorbent filler to interact with impurities in the matrix to adsorb impurities to achieve impurity removal. Specifically, after the homogenized sample is extracted by acetonitrile (or acidified acetonitrile), the salt is separated and layered by extraction salt, the matrix dispersive extraction machine is used, and PSA or other adsorbent and most of the interfering substances in the matrix are used organic Acid, fatty acid, carbohydrate, etc. are combined and removed by centrifugation to achieve purification.

Chemicals And Reagent

- Imidacloprid 17.8%
- Chlorpyrifos 20**%** (local pesticide)
- Acetonitrile
- Formic acid
- Ammonium formate
- Methanol (MeOH)
- Sodium chloride (NaCl)
- Anhydrous magnesium sulphate ($MgSO_4$)
- Sodium sulphate ($Na2SO_4$)
- Graphitized carbon black (GCB) (Sorbend)
- primary, and secondary amine (PSA, 40 μm)
- Millipore water (18.2 MΩ).

Procedure

The pesticide residues were extracted and cleaned up from bitter gourd fruit, by modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method (Anastassiades *et al.*,2003). The flowchart of extraction of pesticide is shown in Figure 3

Take 10g of sample in 50ml of the centrifuge tube

Add 20ml of ACN and shake well by vortex

Add 4$MgSO_4$+1g of NaCl and shake well by vortex

Centrifuge @6000rpm for 10 minutes

Take 6ml of supernatant in prefilled centrifuge tube with 100mg of PSA+600mg of $MgSO_4$+10mg of GCB and shake well by vortex

Centrifuge @ 3000rpm for 10minutes

Take supernatant 4ml and concentrate to near dryness using Turbovap

Final volume made upto 1ml with ACN of HPLC grade (Hexane for GC)

Analysis of samples with GC-MS/LC-MS/MS

APPENDIX-II

LC-MS INSTRUMENTATION (Mawtham *et al.*,2022)

Principle

This method quantifies pesticides using liquid chromatography triple quadrupole mass spectrometry (LC-MS). The compounds of interest are separated using high performance liquid chromatography (HPLC) on a reverse phase C-18 column. After separation, the pesticides are ionized by electrospray ionization and directed into the mass spectrometer.

The triple quadrupole mass spectrometer is operated in dynamic multiple reaction monitoring (dMRM) mode. In dMRM, each compound has a retention time specific time window for acquisition. During acquisition, a precursor ion mass is selected in the first quadrupole, fragmented in the collision cell, compound specific product ions are selected in the last quadrupole, and finally sent to the detector.

Procedure

Detection of imidacloprid residues was made using a single Quadrupole from Shimadzu 2020 series LC-MS containing reverse phase C18 (Eclipse plus- Agilent) column (250 mm length x 4.6 mm id, 5 μm particle size) with the following instrumental parameter

LC-MS SPECIFICATIONS	
DETECTOR	MS detector
SOFTWARE	Shimadzu lab solutions software version 5.6
MOBILE PHASE	A (methanol): B (water) at 70:30 with 2mM ammonium formate with 0.05%formic acid
FLOW RATE	0.5 mL min^{1}
MASS RATIO (M/ Z)	256 and 156 for imidacloprid and 6-CNA
LONIZING MODE	Positive selected ion monitoring(+SIM) and negative ion monitoring (- SIM) mode for imidacloprid and 6- CNA, respectively
INJECTION VOLUME	101
DRYING GAS FLOW	15 L min^{1}rate
COLUMN OVEN	15 min
TEMPERATURE NEBULIZER GAS	1.5 L min1
FLOW RATE LC-MS PUMP PRESSURE	48 kg/cm^{2}
HEAT BLOCK TEMPERATURE	200°C
DESOLVATIONLINE(DL) TEMPERATURE	250°C
CAPILLARY VOLTAGE	3.5 kV
RETENTION TIME OF ANALYTES	5.87±0.02 and 6.76±0.02 min for imidacloprid , ***Chlorpyrifos*** and 6-CNA

PROTEIN DATA BANK

Figure 4.5Web Page of RCSB PDB

Website

The link to accesses the protein for further analysis→ https://www.rcsb.org

Procedure

The enzyme was downloaded from RCSB Protein Data Bank (PDB) and the enzyme was refined before docking. The steps involved are:

- Open Accelrys discovery studio viewer.
- File→Open→Select the enzyme file downloaded from RCSB PDB.
- Click View option and then click Hierarchy.
- Click water molecules.
- Click water molecule →Select all water molecules→cut.
- Select ligand, which is unnecessary and cut.
- Save the molecule in a desired location

PUBCHEM

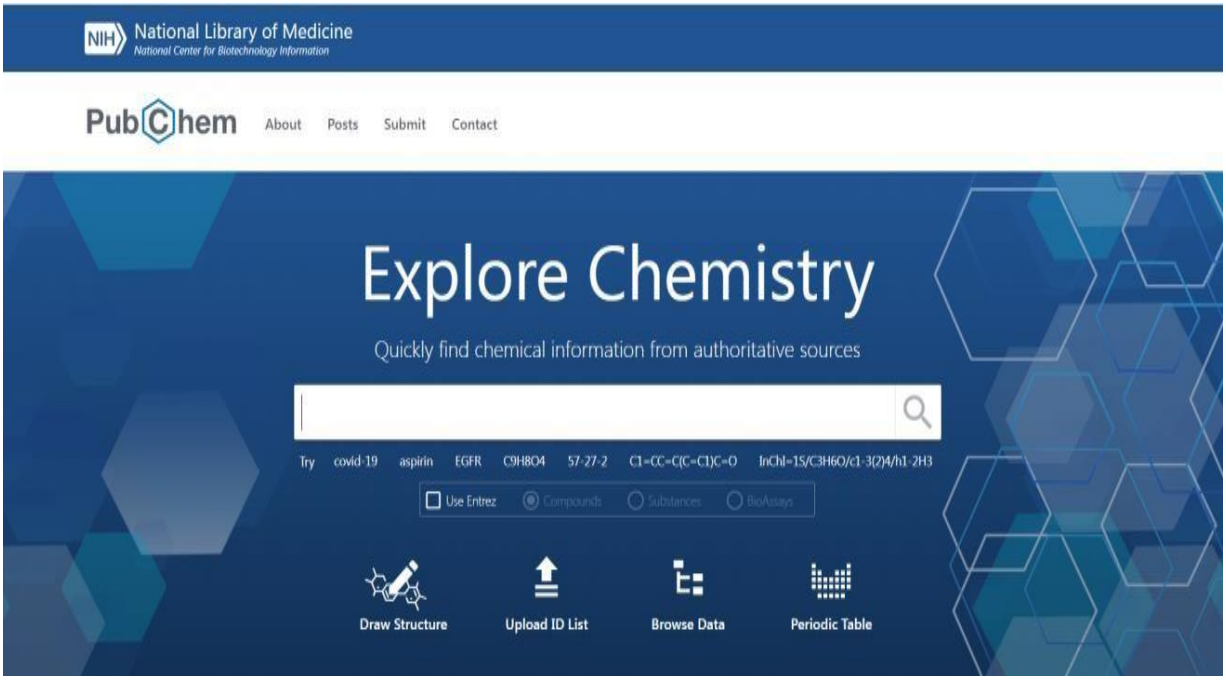


Figure 4.6 Web Page of PUBCHEM

Website

The link to accesses then chemical information for further analysis→ https://pubchem.ncbi.nlm.nih.gov/

Procedure

- Click the pubchem website
- Enter the required compound
- Click the search button to get the ligand file
- The ligand files which are prepared by above said procedures are taken for docking.

APPENDIX-III

DOCKING STIMULATION (Alberg and Schreiber,1993).

Lamarckian genetic algorithm methodology was employed for docking simulations implemented in AutoDock 4.2.8. The standard docking procedure was used for a rigid protein

and flexible ligand whose torsion angles were identified. A grid of 60, 60, and 60 points in x, y, and z directions was built with grid spacing of 0.375 Å. The default settings were used for all other parameters**.**

ANALYSIS AND VISUALIZATION OF DOCKING SIMULATION RESULTS

At the end of the docking, the best poses were analyzed for hydrogen bonding and calculation using Discovery studio 4.2.8.was used to view the structure. From molecular docking study estimated by molecular docking score.

PROCEDURE OF IN SILICO STUDIES

- Softwares and Databases used
- Accelrys accord for excel
- RCSB protein data bank
- PUBCHEM
- Autodock 4.2 which combines
- Autodock tools
- Accerlys discovery studio viewer

AUTODOCK 4.2.8

Automated molecular docking study was conducted with Autodock 4.2.6 (ScrippsResearch Institute, La Jolla, CA). The root of each ligand was detected, and torsion angles were identified for ten independent runs per ligand. A grid box of 126 × 100 × 96 points in x, y and z directions was built with a grid spacing of 0.575 A° for amylase macromolecule. The default settings were used for all other Autodocking parameters. At the end of docking, the best poses were analyzed for binding free energy (kcal/mol), docking predicted inhibition constant (Ki), intermolecular.

AutoDock is a suite of automated docking tools. It has been designed to predict how small l molecules, such as substrates or drug candidates, bind to a receptor of known three dimensional structures. AutoDock 4 comprises of a couple of main programs:

(1) Autodock: This program performs the docking of the ligand to a set of grids describing the target protein;

(2) Autogrid: This program pre-calculates these grids.

In addition to using them for docking, the atomic affinity grids can be visualized. This is useful for organic synthetic chemists to design better binders. AutoDock finds applications in major areas like

(1) X-ray crystallography;

(2) Structure-based drug design;

(3) Lead optimization;

(4) Virtual screening (HTS);

(5) Combinatorial library design;

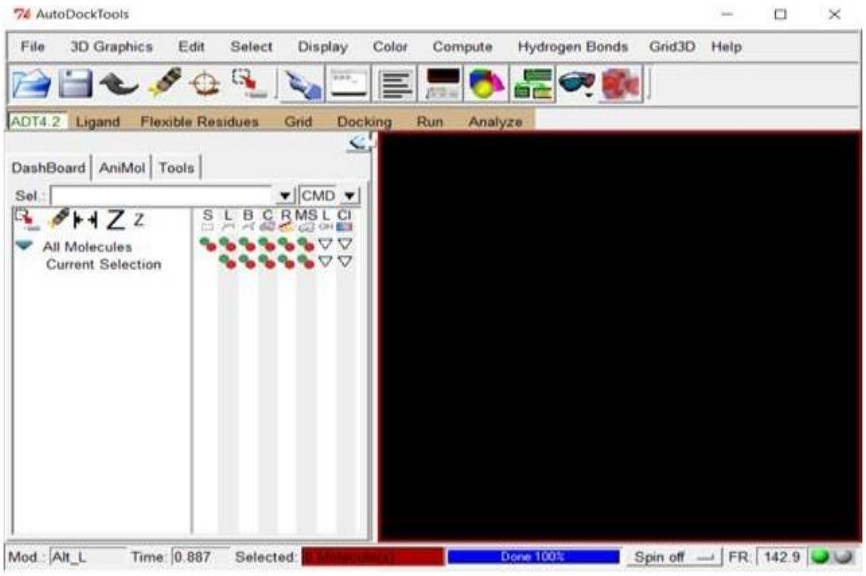
(6) Protein-protein docking;

(7) Chemical mechanism studies.

It is very reliable and gives high quality predictions of ligand conformations. Further, it provides good correlations between predicted inhibition constants and experimental ones (Morris *et al.,*2009). AutoDock has also been shown to be useful in blind docking, wherethe location of the binding site is not known.

Molecular docking

Autodock was used to perform docking of each selected ligand with the target protein. The ligand pose is the integration of position and orientation of a ligand in comparison to the receptor and its conformation in flexible docking (Alberg and Schreiber 1993). A set of previously calculated receptor grids and one or more ligand structures are needed to perform ligand docking process. It is recommended to carry out ligand preparation before docking. The docking process skips a ligand, if a correct Lewis structure cannot be generated for the same. The ligands containing unparametrized elements, such as arsenic, or types of atoms not supported by the OPLS force fields, like explicit lone pair atoms are also automatically skipped. The shape and characteristic of the receptor were represented on a gridby various sets of fields that give progressively more accurate scoring of the ligand poses (Sahoo *et al.,* 2014).



DISCOVERY STUDIO 4.2.8.

Define the protein as the receptor molecule.

- In the Files Explorer, find and double-click to open the 1kim.pdb file.
- The protein will appear in a new molecular window (Figure 1).
- Click to select 1kim in the system view
- In the Tools Explorer, expand Receptor-Ligand Interactions | Define and Edit Binding Site and click "**Define Receptor**".

Look for possible binding regions in the receptor

- If the crystal structure does not include **H** atoms, select "**Chemistry | Hydrogens | Add**" in the menu bar to add hydrogen.
- In the Tools Explorer, expand "**Receptor-Ligand Interactions | Define and Edit Binding Site**", and click "**From Receptor Cavities**" under the "**Define Site**" column.
- Find the possible binding sites in the receptor by looking for the cavity in the receptor.
- Click the "**Input Ligands**" parameter box, select "**Molecule: All**" from the drop-down list, and select all ligand conformations in the Molecule window.
- Click the "**Input Receptor**" parameter box and select "**Molecule:1kim**" from the drop-down list. Expand the "**Input Receptor**" parameter, click the "**Hydrogen Bond**"

parameter box, and select "**True**" from the drop-down list. Expand the "**Hydrogen Bond**" parameter and set the "**Scope**" parameter to "**Residue**" and "**Molecule**".

- The number of hydrogen bonds formed between the docked posture and the entire receptor and each amino acid residue of the receptor will be calculated.

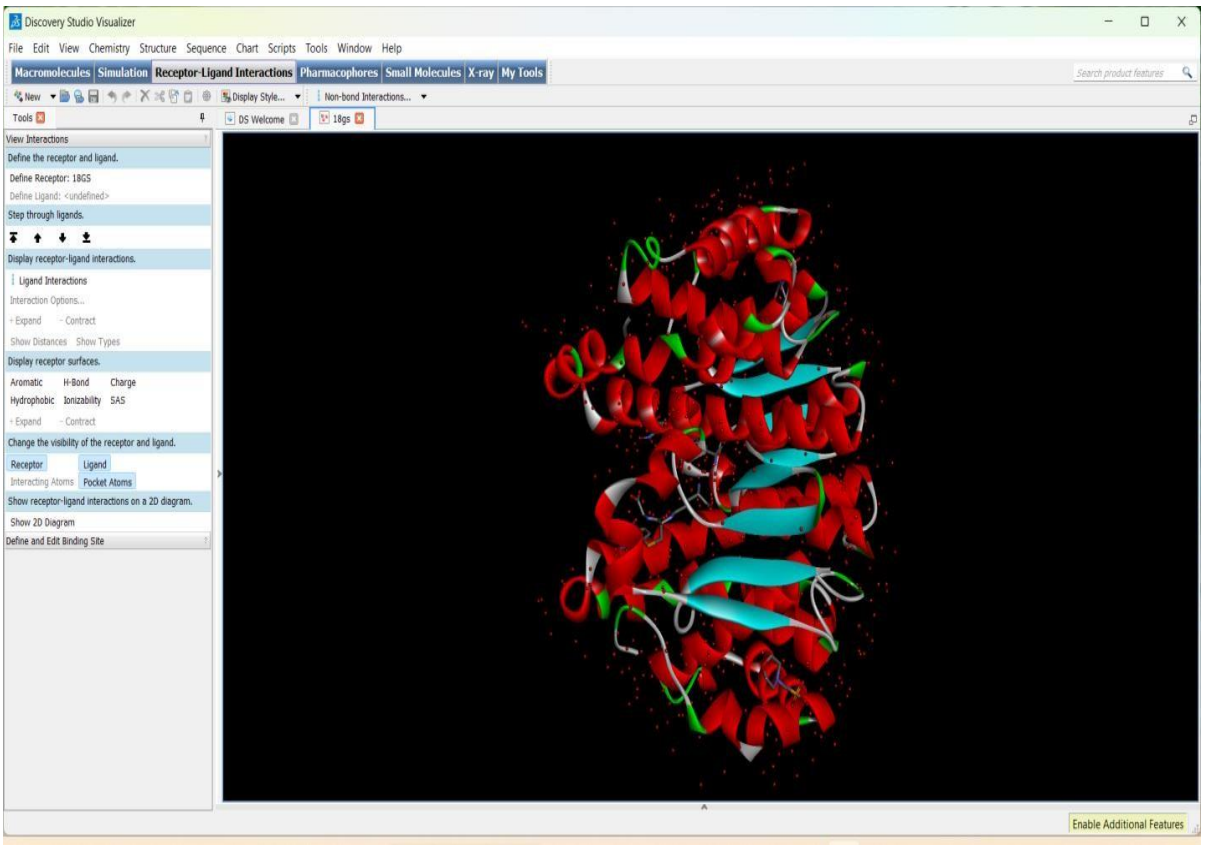


Figure 4.7 Webpage of Discovery Studio