IMPACT OF DIETARY SUPPLEMENTATION OF ASCORBIC ACID ON REPRODUCTIVE GENOTOXICITY IN *APIS CERANA INDICA* F. EXPOSED TO IMIDACLOPRID

Thesis submitted to The University of Calicut

For the award of the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Sciences

By

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Date: 04/11/2023

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DECLARATION

I hereby declare that the work presented in the thesis entitled **"Impact of dietary supplementation of ascorbic acid on reproductive genotoxicity in *Apis cerana indica* F. exposed to imidacloprid"** is a genuine record of research work done carried out by me under the guidance and supervision of **Dr. C.V. Sreeranjit kumar, Associate professor (Retd.), P. G. & Research Department of Zoology, Govt. Victoria College, Palakkad.** To the best of my knowledge, no part of this thesis has been previously submitted for the award of any degree, diploma or associateship in any other University.

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Syama P. S.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
APX	Ascorbate Peroxidase
CAT	Catalase
CCD	Colony Collapse Disorder
G6PD	Glucose 6 Phosphate Dehydrogenase
GPx	Glutathione Peroxidase
GSR	Glutathione Reductase
GST	Glutathione S Transferase
GSH	Reduced Glutathione
GSSG	Oxidised Glutathione
H_2O_2	Hydrogen Peroxide
IMD	Imidacloprid
nAChRs	Nicotinic Acetylcholine Receptors
POD	Peroxidase
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
VIT C	Ascorbic acid
Vg	Vitellogenin

ABSTRACT

The Indian bee, *Apis cerana indica* F., is one of the most important bee species domesticated in India for honey production and crop pollination. It is an effective pollinator with high survivability and is responsible for maintaining global biodiversity and food production. In addition to providing crucial pollination services to agricultural crops, it has significant economic potential for both small- and large-scale beekeeping. Population declines of *A. cerana indica* are affecting ecosystem services and food security. Worker bees are exposed to various stressors while foraging and are vulnerable to pesticides due to a decline in the number of genes encoding key enzyme families responsible for detoxification. Neonicotinoids are one of the major stresses contributing to the decline of honey bee populations. They are a group of systemic insecticides with different application methods that are increasingly used for crop protection. Imidacloprid was the first neonicotinoid introduced to the market and is one of the fastest growing neonicotinoids. Because of its systemic properties, they are highly toxic to the non-target organisms including beneficial insects.

In the present study, it was hypothesized that dietary supplementation with ascorbic acid (VIT C) could reduce the toxicity induced by a sublethal concentration of imidacloprid (IMD) in worker bees of *Apis cerana indica*. To conduct the study, honey bee colonies were provided with sugar syrup for six months for the control group and sugar syrup containing 0.2% VIT C for the experimental group. Worker bees from both groups were randomly collected and exposed to 1µg/mL IMD. The activities of antioxidant enzymes were examined to evaluate the effect of ascorbic acid supplementation on peroxidative damage induced by imidacloprid. The expression of nicotinic acetylcholine receptors (*nAChRα1 and nAChRα2* subunit), which are the targets of imidacloprid, and the activities of acetylcholinesterase (AChE) were analyzed to determine the toxicity of imidacloprid on neuronal transmission. To investigate the possible effects of imidacloprid on reproduction, ovarian expression of the multifunctional gene *vitellogenin* and expression of *CYP336A1* were analyzed. Histological analysis was performed to determine the effect of ascorbic acid supplementation on the modification of cyto architecture of midgut and ovarian tissues on exposure to imidacloprid.

The activities of antioxidant enzymes including catalase, superoxide dismutase, glutathione S-transferase and glutathione peroxidase in the hemolymph and midgut tissues of worker bees were significantly decreased by exposure to IMD as a single agent. However, their activities showed a significant increase when supplemented with ascorbic acid. When worker bees were exposed to imidacloprid (IMD) alone, there was a significant decrease in AChE activity and a significant upregulation of *nAChRα1* and *nAChRα2* subunit expression in brain tissue. Our results indicated that the dietary supplementation with ascorbic acid has the potential to maintain AChE activity and expression of nAChRs at normal levels to some extent against the toxic effects of imidacloprid. The expression of *vitellogenin* and *CYP336A1* was found to be down-regulated in the ovaries of queen bees upon exposure to IMD, while the supplementation of ascorbic acid maintained the ovarian expression of *vitellogenin* and *CYP336A1* at normal levels. Histological examination of midgut tissue revealed cytoarchitectural alterations and rupture of the peritrophic membrane in workers and queen bees exposed to IMD. The damage was alleviated and the peritrophic membrane was intact in worker bees treated with ascorbic acid. Histological examination of the ovaries of queen bees showed degeneration of oocytes and trophocytes upon exposure to IMD, whereas no complete degeneration was observed in queen bees supplemented with ascorbic acid.

Our results suggest that dietary supplementation with ascorbic acid is able to maintain the redox status of tissues and to defend against the peroxidative damage induced by the sublethal concentration of imidacloprid. Ascorbic acid has the potential to maintain the redox status in the ovary, as shown by the upregulation of *vitellogenin* and *CYP336A1*, which protect the organism from the oxidative damage induced by imidacloprid. Ascorbic acid supplementation may provide protection against peroxidative damage induced by sublethal concentrations of imidacloprid, as evidenced by the maintenance of normal tissue morphology. Therefore, we propose that ascorbic acid can be used as an effective supplement to alleviate the toxic aspects of the pesticide-induced oxidative stress.

സംഗ്രഹം

തേൻ ഉത്പാദനത്തിനും, വിള പരാഗണത്തിനും വേണ്ടി ഇന്ത്യയിൽ വളർത്തുന്ന പ്രധാന തേനീച്ചകളിൽ ഒന്നാണ് *Apis cerana indica* F. ആഗോള ജൈവ വൈവിധ്യത്തിന്റെ പരിപാലനത്തിനും, ഭക്ഷ്യ ഉത്പാദനത്തിനും ഇവ അത്യന്താപേക്ഷിതമാണ്. തേനീച്ചകളുടെ എണ്ണം കുറയുന്നത് ആവാസവ്യവസ്ഥയുടെ സേവനങ്ങളെയും ഭക്ഷ്യസുരക്ഷയെയും, കാർഷിക മേഖലയെയും പ്രതികൂലമായി ബാധിക്കുന്നു. ആവാസവ്യവസ്ഥയുടെ നാശം, പരാന്നഭോജികളുടെയും, രോഗാണുക്കളുടെയും ആക്രമണം, കാർഷികമേഖലയിലെ അശാസ്ത്രീയമായ രസവസ്തുക്കളുടെ ഉപയോഗം എന്നിങ്ങനെയുള്ള ഒന്നിലധികം ഘടകങ്ങൾ തേനീച്ചകളുടെ എണ്ണത്തെ പ്രതികൂലമായി ബാധിക്കുന്ന ഘടകങ്ങളാണ്. ഇവയിൽ ഒരു പ്രധാന ഘടകമാണ് നിയോനിക്കോട്ടിനോയ്ഡ് എന്ന വിഭാഗത്തിൽ പെടുന്ന കീടനാശിനികൾ. വിപണിയിൽ എത്തിയ ആദ്യത്തെ നിയോനിക്കോട്ടിനോയിഡും, കാർഷികമേഖലകളിൽ കൂടുതലായി ഉപയോഗിച്ച് വരുന്ന നിയോനിക്കോയിഡുകളിലൊന്നുമാണ് ഇമിഡാക്ലോപ്രിഡ്. വിള സംരക്ഷണത്തിനായി ഉപയോഗിക്കുന്ന ഇവ ചെടികളുടെ വിവിധ ഭാഗങ്ങളിലും, പൂമ്പൊടി, പൂന്തേൻ എന്നിവയിലും എത്തുന്നതിലൂടെ തേനീച്ചകളുടെ ആരോഗ്യത്തിന് ഗുരുതരമായ ഭീഷണിയാകുന്നു.

ഇമിഡാക്ലോപ്രിഡിന്റെ വിഷാംശം മൂലം തേനീച്ചയിൽ ഉണ്ടാകുന്ന ആരോഗ്യപ്രശ്നങ്ങൾ കുറക്കുന്നതിന്, വിറ്റാമിൻ-സി (അസ്കോർബിക് അമ്ലം) സംപുഷ്ടപ്പെടുത്തിയ ഭക്ഷണത്തിനു കഴിയുമെന്ന അനുമാനമാണ് ഈ പഠനം മുന്നോട്ട് വക്കുന്നത്. ഈ പഠനത്തിനായി *A. cerana indica* തേനീച്ച കോളനികൾ രണ്ടായി തരംതിരിച്ച്, നിയന്ത്രിത വിഭാഗത്തിന് പഞ്ചസാര ലായനിയും, പരീക്ഷണ വിഭാഗത്തിന് വിറ്റാമിൻ-സി ഉൾപ്പെടുത്തിയ പഞ്ചസാര ലായനിയും നൽകി പരിപാലിച്ചു. രണ്ടു വിഭാഗങ്ങളിലെയും തേനീച്ചകളെ അസംഗതമായി ശേഖരിച്ച് Control, IMD, VIT C, VIT C+IMD എന്നിങ്ങനെ നാലു വിഭാഗങ്ങളായി തിരിക്കുകയും ഇമിഡാക്ലോപ്രിഡ് പ്രയോഗിക്കുകയും ചെയ്തു. ഇമിഡാക്ലോപ്രിഡ് മൂലമുണ്ടാകുന്ന ആരോഗ്യപ്രശ്നത്തിൽ വിറ്റാമിൻ-സിയുടെ പ്രഭാവം വിലയിരുത്തുന്നതിന് ആന്റിഓക്സിഡന്റ് രാസാഗ്നികളുടെ പ്രവർത്തനം പഠിച്ചു. നാഡീവ്യവസ്ഥയിൽ

ഇമിഡാക്ലോപ്രിഡിന്റെ പ്രഭാവം നിർണയിക്കാൻ nAChRs ന്റെ പ്രകടനവും, AChE രാസാഗ്നിയുടെ പ്രവർത്തനവും വിശകലനം ചെയ്തു. പ്രത്യുല്പാദനത്തിൽ ഇമിഡാക്ലോപ്രിഡിന്റെ വിഷാംശം മൂലമുണ്ടാകുന്ന പ്രശ്നങ്ങൾ പഠിക്കുന്നതിനും, വിറ്റാമിൻ-സി യുടെ സാധ്യമായ പ്രഭാവം പഠിക്കുന്നതിനുമായി അണ്ഡാശയത്തിലെ *CYP336A1*, *vitellogenin* എന്നീ ജീനുകളുടെ പ്രകടനവും, അണ്ഡാശയകലകളിൽ ഉണ്ടാകുന്ന മാറ്റങ്ങളും വിശകലനം ചെയ്തു.

ഇമിഡാക്ലോപ്രിഡ് പ്രയോഗിച്ചപ്പോൾ ആന്റിഓക്സിഡേറ്റീവ് രാസാഗ്നികളുടെയും, AChE രാസാഗ്നിയുടെയും പ്രവർത്തനം ഗണ്യമായി കുറയുന്നതായും, വിറ്റാമിൻ-സി യുടെ പ്രഭാവത്തിൽ ഇവയുടെ പ്രവർത്തനങ്ങൾ കൂടുന്നതായും ഈ പഠനത്തിലെ ഫലങ്ങൾ കാണിച്ചു. nAChRs ന്റെ പ്രകടനത്തെയും, *CYP336A1, vitellogenin* എന്നീ ജീനുകളുടെ പ്രകടനത്തെയും ഇമിഡാക്ലോപ്രിഡ് പ്രതികൂലമായി ബാധിക്കുന്നുണ്ടെന്നും, എന്നാൽ വിറ്റാമിൻ-സി യുടെ പ്രഭാവത്തിൽ ഇവയുടെ പ്രകടനത്തെ സാധാരണ നിലയിൽ നിലനിർത്താനുള്ള കഴിവുണ്ടെന്നും ഫലങ്ങൾ സൂചിപ്പിക്കുന്നുണ്ട്. ഇമിഡാക്ലോപ്രിഡ് മൂലം തേനീച്ചകളുടെ വ്യത്യസ്ഥ കലകളിൽ ഉണ്ടാകുന്ന nAChRs ന്റെ പ്രകടനത്തെയും, *CYP336A1, vitellogenin* എന്നീ ജീനുകളുടെ പ്രകടനത്തെയും ഇമിഡാക്ലോപ്രിഡ് പ്രതികൂലമായി ബാധിക്കുന്നുണ്ടെന്നും, എന്നാൽ വിറ്റാമിൻ-സിയുടെ പ്രഭാവത്തിൽ ഇവയുടെ പ്രകടനത്തെ സാധാരണ നിലയിൽ നിലനിർത്താനുള്ള കഴിവുണ്ടെന്നും ഫലങ്ങൾ സൂചിപ്പിക്കുന്നുണ്ട്. ഇമിഡാക്ലോപ്രിഡ് മൂലം തേനീച്ചകളുടെ കലകളുടെ ഘടനാതലത്തിൽ ഉണ്ടാകുന്ന വ്യതിയാനങ്ങളെ വിറ്റാമിൻ-സി യുടെ പ്രഭാവത്തിൽ ലഘൂകരിക്കപ്പെടുന്നുണ്ടെന്നും ഈ പഠനം വ്യക്തമാക്കുന്നു. കലകളിലെ റീഡോക്സ് നില നിലനിർത്തുന്നതിനും, ഇമിഡാക്ലോപ്രിഡ് മൂലമുണ്ടാകുന്ന പ്രതികൂല ആരോഗ്യപ്രശ്നങ്ങൾ പ്രതിരോധിക്കുന്നതിനും വിറ്റാമിൻ-സിക്ക് കഴിയുമെന്ന് ഈ പഠനത്തിന്റെ ഫലങ്ങൾ തെളിയിക്കുന്നു. ആയതിനാൽ കീടനാശിനിമൂലമുണ്ടാകുന്ന വിഷ വശങ്ങൾ ഒഴിവാക്കുന്നതിന് വിറ്റാമിൻ-സി ഉപയോഗിക്കാമെന്ന് ഞങ്ങൾ ഈ പഠനത്തിന്റെ ഫലങ്ങളുടെ അടിസ്ഥാനത്തിൽ നിർദേശിക്കുന്നു.

Chapter 1.
Introduction

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Chapter 1. Introduction

1.1. Apiculture, the art of beekeeping

Kerala is blessed with a diverse floral landscape and pleasant climatic conditions that provide ample opportunities for beekeeping in the state, which accounts for a significant share of India's total honey production. Coconut palms are grown on about 9.3 lakh hectares of land in Kerala, and are the major source of pollen for bees. Rubber plantations cover 5.5 lakh hectares of land in Kerala, and provide a good source of nectar for bees (Khurana and Kumar 2020). *Apis cerana* beekeeping is widespread and practiced by individuals in Karnataka, Kerala and Tamil Nadu, and large quantities of honey are obtained from rubber plantations. Kerala and Tamil Nadu have traditionally been the leading states in beekeeping, with Kerala accounting for 70% of India's annual honey production (Chutani and Singh, 2018). In Kerala, beekeeping has traditionally been practiced as an indigenous industry, not only for food production but also as a component of traditional medicine (Gupta *et al.,* 2014).

Beekeeping is a branch of economic entomology that involves the rearing and management of honey bees for the production of the honey and various bee products, i.e. royal jelly, bee pollen, bee propolis, bee venom, queen bees, etc. (Chand *et al.,* 2021). Beekeeping is a suitable occupation for a huge number of rural people, miners, tribals, horticulturists, agriculturists and others. The honey, beeswax, and pollination services of bees, which increase the yield of many agricultural and horticultural crops, are the most valued outputs of the sector (Sain and Nain, 2017). Beekeeping supports livelihoods, protects the environment, enhances agriculture, and ensures food security. Over 100,000 plant species would be extinct without the pollination of bees (Agera, 2011). Honey bees can be used to pollinate agricultural and horticultural crops, as well as to produce hybrid seeds for vegetables and other bee pollination technologies for the production of various products (Thomas *et al.,* 2002).

Apart from food and beverages, honey and other hive products are an inseparable part of a variety of cosmetics and pharmaceutical items (Dunne *et al.,* 2021). Royal jelly, a protein-rich glandular secretion supplied by bees to nourish larvae, is commonly used as a basic ingredient in many specialized products for human health and beauty (Bagameri *et*

al., 2022). Honey is supposed to promote immune defense, and due to its excellent medicinal properties, the demand for honey increased in 2020 due to the COVID -19 pandemic (Hossain *et al.,* 2020).

Honey bees are the most important and economically significant group of pollinators in temperate climates and are responsible for global food production (Blacquiere *et al.,* 2012). There are 26 species defined based on morphometric, behavioral, and biogeographic characteristics (Yadav *et al.,* 2017). The predominant bees include *Apis mellifera*, *A. dorsata*, *A. florea*, *A. cerana*, and *Tetragonula irridipennis* (stingless bees). Due to their breeding habits, *A. mellifera* L. and *A. cerana indica* F. (Indian honey bee) are extensively maintained and transported by beekeepers (Sain and Nain, 2017).

A. mellifera occurs naturally in a variety of climates and can withstand excessive heat and aridity. In temperate zones, its colonies are found from sea level to 1000 m above sea level, while in the tropics they are found from sea level to 3700 m (Dutton *et al.,* 1981). *A. dorsata* occurs in the Indian subcontinent, Pakistan, and Sri Lanka, as well as parts of Indonesia and the Philippines (Gupta, 2014). *Apis florea* occurs mainly in warm climates such as Pakistan, Iran, Oman, India, and Sri Lanka, but is also found in tropical forests and agricultural areas. Its range is essentially not above 1500 m, but it can be sighted in tropical forests and agricultural areas (Yadav *et al.,* 2017). *A. cerana* has been found in tropical, subtropical, and temperate regions of Asia, with populations ranging from the Indian subcontinent and Sri Lanka in the west to Indonesia and the Philippines in the east.

In India, beekeeping with *A. cerana* has long been practised for honey production and pollination services. Farmers traditionally keep this bee species in log, wall and box hives (Singh, 2014). The co-evolution of local floral supply, pests and predators has made *A. cerana* an effective pollinator with high survivability (Thakur, 2012). *A. cerana* not only provides important pollination services for agricultural crops, but also has significant economic potential for small and large-scale beekeeping (Koetz, 2013). *A. cerana* colonies are easily expanded and propagated by splitting strong colonies and can be maintained without additional supplements (Abrol, 2013). Successfully reproducing colonies that are well managed can be sold (Verma and Attri, 2008). Adaptability to local conditions, convenience of maintenance, resistance to pests and diseases, honey quality

and pollination performance are the advantages of *A. cerana.* The technical challenges faced by beekeepers are absconding and swarming behaviour, limited honey yield per colony, susceptibility to Thai Sac Brood Virus, and sensitivity to various diseases and pests (Koetz, 2013; Wendorf, 2002).

1.2. Apis cerana indica F.

Apis cerana indica F., is one of the most important bee species domesticated in India for honey production and crop pollination (Yadav *et al.,* 2017). There are several ecotypes with a wide range of morphological characteristics. It can be found in a wide range of environments, from cool sites at higher latitudes and altitudes to arid, semi-arid habitats and tropical climates (Ruttner, 1988). Colonies of *A. cerana indica* consist of three types of adult bees: a queen, several thousand workers, and a few hundred drones (Radloff *et al.,* 2010). The queen is the only reproductive female in the colony and is the most important regulatory factor for colony functions (Winston, 1991). Workers are the sterile females that perform hive maintenance tasks such as cleaning the hive, caring for the brood, collecting pollen and nectar, honey making as well as defending the colony against enemies. Drones are the male bees in the colony, present only in late spring and summer (Koetz, 2013).

In honeybees there is a division of labour or polyethism. Polyethism in honey bees is age-dependent, and each individual assumes a role in the hive appropriate to its physiological state, which changes with its tasks throughout life (Smith *et al.,* 2022). For newly emerged adult worker bees, roles in the colony begin as "nurse bees" (3-12 days old) caring for larvae and the queen. The developing larvae are cared for by the nurse bees, who feed them a mixture of honey, pollen, and royal jelly (Standifer, 1980). The role of worker bees then shifts to "house bees" (13-20 days old), which perform tasks such as cleaning cells, caring for nestmates, food processing, and comb building. They clean themselves and each other, remove infected brood, and maintain hygiene in the colony (Koetz, 2013). Food processing is an important task of worker bees. They use it as food for the growing larvae and also need it as food for the winter (Moritz and Crewe, 2018). New combs are built using the wax flakes produced by the wax glands under the abdomen of worker bees (Oliver, 2021). Finally, the role of the worker bee shifts to that of a "forager" bee (> 21 days), which takes on the riskiest tasks of guarding and gathering resources. Each forager bee in a colony must work to collect nectar from

flowers and turn it into honey in the hive (Seeley, 2009). They perform unique dances (waggle dance and round dance) to guide other forager bees to good nectar and pollen sources (Steffan-Dewenter and Kuhn, 2003). They guard their resources (honey and young bees) and defend their colonies by stinging anything that might threaten their safety (Radloff *et al.,* 2010).

As social insects, honey bees have a particular life history that involves a dynamic system of tasks in which each individual bee functions as a superorganism and its existence correlates with the success of the colony (Berenbaum and Liao, 2019). The maintenance of the social structure of the bee colony, the communication between the queen, workers and drones in the bee colony, and the overall integration of activities necessary for the survival of the bee colony are regulated by the distribution of chemical pheromones among the members of the bee colony (Trhlin and Rajchard, 2011).

During seasonal mating flights, drones mate with the virgin queen, release their sperm, and die immediately after mating (Harano and Sasaki, 2013). During the nuptial flight, the queen mates with several drones and stores the sperm in her spermatheca. After mating, the queen returns to the hive and begins laying fertilized or unfertilized eggs in the hive (Seeley, 2014). Each time the queen lays an egg in the worker cell, she releases sperm from the spermatheca and produces fertilized eggs. The fertilized eggs may give rise to workers or a new generation of queens, depending on the food they receive in the early stages of larval development (Woyke, 1963). The queen doesn't release sperm when her egg is laid in a larger drone cell, resulting in the development of unfertilized eggs. The unfertilized eggs may develop into male (haploid) drones (Seeley, 2014).

Worker honey bees raise a new queen under three different circumstances: emergency, supersedure or swarming. When an existing queen dies, is lost or removed, or is unable to lay eggs, worker bees select younger worker larvae to produce emergency queens (Wenseleers and Van Oystaeyen, 2011). The queen is reared in large finger-shaped queen cells built vertically into the lower part of the combs. Larvae selected to become future queens develop in the queen cells by consuming royal jelly regularly throughout their development. Workers and drones are fed exclusively royal jelly for the first three days of their larval life, after which they receive worker jelly, a mixture of royal jelly, pollen, and nectar (Khan *et al.,* 2021).

Royal jelly is a sweet, protein-rich secretion synthesized by the hypopharyngeal glands in the head of worker bees (Kamakura, 2012). The hypopharyngeal glands are age-related paired glands consisting of small oval bodies, the acini and secretory ducts. The activity of the hypopharyngeal glands depends on the size of the acini, which are fully developed in young workers (6-13 days old) and have large functional secretory acini. When workers begin foraging, the hypopharyngeal glands shrink, degenerate and become less productive (Deseyn and Billen, 2005). These glands are also susceptible to many conditions, including malnutrition, heat, and varroa infestation, which can cause degeneration of the glands (Yousef *et al.,* 2014; Faita *et al.,* 2018).

When collecting pollen and nectar, *A. cerana* pollinates flowers, which is the most important ecological service provided by them (Partap, 2011). They generally prefers to forage within 200-300 m of its nest, and its maximum range is 1500-2500 m (Dyer and Seeley, 1991). *A. cerana* pollinate flowers over a longer period of time because they start foraging earlier in the day and stop later (Li *et al.,* 2021). They have comparatively more pollen collectors than nectar collectors (Verma and Partap, 1994). Therefore, they are considered excellent pollinators of a wide range of fruit and vegetable crops in Asia (Gautam *et al.,* 2022). *A. cerana* is able to thrive in disturbed landscapes and is an important canopy pollinator in the rainforests of the Western Ghats and Sri Lanka (Corlett, 2004).

1.3. Pollinator Decline

The diversity of flowering plants and the sustainability of the ecosystem are maintained by pollination. Insect pollinators, especially bees, are directly responsible for the production of 84% of the crop species grown in Europe (Williams 1994). Globally, pollinators are considered necessary for 70% of the 124 major crops grown for direct human consumption. Insect pollination is both an ecosystem service and a production method commonly used by farmers (Williams, 1994; Klein *et al.,* 2007). Honey bees pollinate 71 of the top 100 crops, accounting for 90% of global food production, making them the most economically powerful pollinator (Gallai *et al.,* 2009). It is estimated that 70% of about 100 crop species that account for 90% of global food production are pollinated by bees and other native insects. Bees pollinate 80% of natural plants and 84% of crops in Europe, representing an annual value of 22,000 million euros. Their

contribution to pollination for the rest of the world is estimated worth 265,000 million dollars per year (Ranz, 2020).

Declines in honey bee populations are increasingly documented all around the world, with potentially devastating consequences in many agricultural areas (Gallai *et al.,* 2009). Several interacting factors such as exposure to pesticides, infestation with parasites and pathogens and habitat loss have been cited as chronic sublethal stresses associated with the decline in honey bee population (Bryden *et al.,* 2013). Reduced pollination can lead to extinction of plant species, decline of fruit- and seed-eating animals, reduction of vegetation cover, and ultimately loss of a healthy ecosystem and its supporting services. In agriculture, inadequate pollination can lead to malformed fruits and a decline in crop yields (Partap, 2011; Indhu *et al.,* 2022).

Colony collapse disorder (CCD), first described in the United States in 2006, is a condition characterized by the rapid loss of adult worker honey bees (Johnson, 2010) that abandon their hives, leaving behind extra honey and pollen stores and causing decline in population (America P. A. N. 2012). CCD is most likely the result of a combination of factors, including sublethal pesticide exposure and the spread of pathogens. This can affect colony fitness and make affected colonies more susceptible to CCD (VanEngelsdorp *et al.,* 2010). Pathogens, poor nutrition, pesticides, parasites and poor management are the main stress factors in honey bee colonies (Parveen *et al.,* 2022). Among these, pesticides are crucial for honey bee population decline, both directly and in combination with the other two main factors such as pathogens and poor nutrition (America P. A. N. 2012). Because of their unexpected effects on non-target creatures, such as honey bees, pesticides pose a serious threat to beekeepers. Chlorothalonil (fungicide), imidacloprid (insecticide), and amitraz (acaricide) have been frequently identified and found in varying concentrations in honey, pollen, brood and honey bees (Tome *et al.,* 2020).

The *A. cerana* populations worldwide have declined due to multiple direct and indirect factors (Theisen-Jones and Bienefeld, 2016). Populations of *A. cerana* have declined sharply in all Asian countries due to habitat loss, climate change, sacbrood virus infection and other agricultural practises. Overuse of pesticides is a major anthropogenic factor responsible for the decline in honey bee populations (Smith *et al.,* 2013). Pesticides affect the behaviour, cognitive processes, and biochemical and

physiological processes of honey bees (Pervez and Manzoor, 2022). Neonicotinoids have been singled out from all the stressors causing decline in bee populations (Godfray *et al.,* 2014). Neonicotinoids, the most widely used systemic insecticides worldwide, have been identified as one of the risk factors for Colony Collapse Disorder (CCD) (Farooqui, 2013).

Inadequate nutrition is another important risk factors of honey bee colony failure. It results from a number of reasons, including decreased foraging opportunities brought on by changing agricultural landscapes or excessive honey collection by beekeepers (Brodschneider *et al.,* 2010; vanEngelsdorp *et al.,* 2010; Brodschneider and Crailsheim, 2010). Beekeepers remove honey storage and replace them with sucrose solution. Vitamins and other micronutrients are completely ignored during the supplementation process (Brown *et al.,* 2022).

1.4. The Neonicotinoids

India, primarily being an agro based country; efforts are being made to increase crop production through intensive agricultural practices. The severity of the problem is so alarming that extensive use of synthetic pesticides has become an integral part of our agricultural system (Casida and Durkin, 2017). Neonicotinoids are systemic insecticides that are highly effective for the control of piercing and sucking insects and are used in agriculture and in home and garden products because of their high performance and relative safety (Lawrence and Sheppard, 2013).

Since their introduction in the 1990s, neonicotinoids have become the fastest growing insecticide class in the history of synthetic pesticides (Jeschke *et al.,* 2011). With no known pesticide resistance in target pests, neonicotinoids have become the most extensively used class of insecticides worldwide (Simon-Delso *et al.,* 2015). Their physicochemical properties offer numerous advantages over organophosphates, carbamates, and pyrethroids (Bonmatin *et al.,* 2015). Neonicotinoids, which include imidacloprid, acetamiprid, clothianidin, thiamethoxam, thiacloprid, dinotefuran, and nitenpyram, are broad-spectrum insecticides with contact and residual activity (Blacquiere *et al.,* 2012). Imidacloprid, clothianidin or thiamethoxam are used for seed treatments of cotton, corn, sunflower, cereals, and vegetables and are absorbed through the roots and distributed throughout the plant (Simon-Delso *et al.,* 2015). Some

formulations are used as foliar sprays for pest control and some as veterinary medicines for flea control (Watts, 2011).

Neonicotinoids are water soluble and can reach plant parts, nectar, and pollen due to their systemic nature, posing a serious threat to honey bee health (Goulson *et al.,* 2013). While foraging, bees near agricultural fields are exposed to various pesticides at harmful levels through multiple pathways (Krupke *et al.,* 2012). Neonicotinoid pesticides have been found in soil, dust, exhaust from planters, water (guttation) droplets exuded by treated plants, and nearby untreated plants and fields (Bonmatin *et al.,* 2015). Because they are systemic insecticides, their traces are found in nectar and pollen, bee products such as bee bread, honey and beeswax (Blacquiere *et al.,* 2012). Neonicotinoids have been detected in pollen collected by bees, exposing developing bees to the pesticides (Krupke *et al.,* 2012). Neonicotinoid insecticides and their transformation products have been detected in honey samples, pollen samples and in the body of honey bees (Codling *et al.,* 2016). Neonicotinoids and their metabolites are highly persistent in soil, aquatic sediments and water.

Neonicotinoids have a variety of adverse sublethal effects on honey bee colonies at field realistic doses, affecting colony performance by impairing foraging success, brood and larval development, memory and learning, central nervous system damage, disease susceptibility, hive hygiene, etc. (Van der Sluijs *et al.,* 2013). The toxicity of neonicotinoids is enhanced by a variety of other agrochemicals, and they interact with infectious pathogens such as *Nosema ceranae* to cause colony collapse (Lu *et al.,* 2020).

Neonicotinoids are synthetic analogs of nicotine that mimic the action of the main excitatory neurotransmitter acetylcholine in the brain of honey bees (Casida *et al.,* 2013). They target nicotinic acetylcholine receptors (nAChRs) and disrupt the functioning of the central nervous system by overstimulation (Matsuda *et al.,* 2001). Electrophysiological and molecular methods have identified several nAChR subtypes with different affinities for neonicotinoid pesticides (Taillebois *et al.,* 2018). Their affinity for these receptors, as well as the neuronal response they elicit after binding to them, is determined by a variety of factors, including their chemical structure, the nAChR and the developmental stage of the insect (Cabirol and Haase, 2019).

These groups of pesticides have been shown to cause behavioural problems such as impaired or delayed communication, navigation, homing and foraging (Henry *et al.,* 2012) and impair their immunity (Di Prisco *et al.,* 2013). The sublethal effects of neonicotinoids on their behavior have been identified as a contributing factor in the decline of their population (Cabirol and Haase, 2019). Neonicotinoids decrease the amount of acetylcholine secreted in brood food, shrink the hypopharyngeal gland, and cause developmental problems in the colony (Grunewald and Siefert, 2019). Neonicotinoids are tasteless and odourless substances that enhance the preference of honey bees for them. This increases the chance of the bee colony being exposed to pesticides, especially nitro-substituted neonicotinoids such as imidacloprid and clothianidin (Sowmiya *et al.,* 2022).

Neonicotinoids have adverse effects on population dynamics and long-term sustainability of both domesticated and wild pollinators (Siviter *et al.,* 2021; Blacquiere *et al.,* 2012). Sublethal exposure to neonicotinoids can damage mitochondrial DNA (mtDNA) and could be a potential cause of colony collapse disorder (Lu *et al.,* 2020). Recent reports have shown that neonicotinoids are neurotoxic to mammals, especially humans, with mitochondria as one of their main targets, disrupting mitochondrial Ca2+ homeostasis and mitochondrial respiration and generating reactive oxygen species (ROS). They can also cause DNA damage, apoptosis, protein oxidation and lipid peroxidation in non-target organisms (Xiao *et al.,* 2022).

1.5. The imidacloprid

Imidacloprid (IMD), a nitro-group containing neonicotinoid, was the first neonicotinoid introduced for effective pest control. It is a chloronicotinyl pesticide that acts by interacting with nicotinic acetylcholine receptors in insects (Watts, 2011). It is used in about 120 countries, and is one of the most popular pesticides on the market (America, P. A. N. 2012). It is effective against aphids, scale insects, whiteflies, jassids, thrips, mealybugs, leaf miners, termites, grasshoppers, shoot flies, mustard sawfly etc. (Decourtye *et al.,* 2004). Imidacloprid is widely used for the control of insect pests because of its multiple applications and lower toxicity to non-target organisms (Medrzycki *et al.,* 2003). Imidacloprid has a different mechanism of action than conventional insecticides and provides excellent control over insects that are resistant to

many other pesticides. It has good residual activity after foliar application, is very photostable, and has satisfactory resilience against rain (Elbert *et al.,* 1991). Imidacloprid has significant leaching potential, which causes them to frequently contaminate groundwater and surface water. In soil, aquatic sediments, and water, imidacloprid and its metabolites can survive for long periods of time (Gupta *et al.,* 2002).

Imidacloprid is highly toxic to bees and has a greater affinity for nicotinic acetylcholine receptors (nAChRs) in the bee brain than nicotine (Nauen *et al.,* 2001). Binding to nAchRs, interferes with signal transduction by overstimulation and prevents the action of acetylcholine (Matsuda *et al.,* 2001). The inability of acetylcholinesterase (AChE) to hydrolyze IMD prolongs the duration of stimulation (Belzunces *et al.,* 2012). This can alter a wide range of behaviors required for the proper functioning of the colony (Siviter *et al.,* 2018). Imidacloprid and its metabolites have been detected in pollen and honey samples (Codling *et al.,* 2016) and also in the hemolymph and body parts of honey bees such as midgut and rectum (Suchail *et al.,* 2004).

Imidacloprid in sublethal doses has long-term effects on honey bees. The route of exposure of imidacloprid determines toxicity, with oral ingestion being more toxic than contact (Blacquiere *et al.,* 2012). Sublethal effects manifested in bees following imidacloprid exposure include inhibition of associative learning (Decourtye *et al.,* 2004), abnormal foraging behavior (Yang *et al.,* 2008), reduction in mobility, and loss of communication ability (Medrzycki *et al.,* 2003). Regarding metabolic effects, imidacloprid impairs brain metabolism (Decourtye *et al.,* 2004) and decreases mitochondrial activity (Nicodemo *et al.,* 2014). Under laboratory conditions, a reduction in mobility and loss of communication ability was observed upon exposure to various concentrations (Medrzycki *et al.,* 2003). Under semi-field conditions, IMD exposure to contaminated flowers showed abnormal foraging behavior in bees (Yang *et al.,* 2008) and impaired olfactory associative behavior (Yang *et al.,* 2012; Li *et al.,* 2019). Sublethal effects of IMD at low concentrations impaired the parts of the brain involved in associative and contextual memory (Decourtye *et al.,* 2004).

Healthy queens are important for the proper functioning of the colony (Amiri *et al.,* 2017). Even if they are not directly exposed to sources contaminated with imidacloprid, they may be affected by wax or royal jelly contaminated with imidacloprid, and nurse bee brood behavior (Dively *et al.,* 2015). Imidacloprid exposure can affect

sperm viability in honey bee queens and inhibit the expression of antioxidant, immunity, and developmental genes in queens (Chaimanee *et al.,* 2016). The female-specific egg yolk precursor vitellogenin, synthesized in the fat body, plays an important role in honey bee reproduction (Cardoso-Junior *et al.,* 2006) and can be used as a suitable biomarker for assessing the extent of energy stress and sublethal effects of pesticides on honey bees (Abbo *et al.,* 2016). It functions as a reproductive protein and also plays a key role in hormone signaling, behavioral maturation, stress resistance and longevity (Cardoso-Junior *et al.,* 2021; Christen *et al.,* 2019). From the fat body, vitellogenin is released into circulating hemolymph and expressed in tissues with vitellogenin receptor protein (Dohanik *et al.,* 2018). In honey bees, vitellogenin is detected in the fat body and ovaries of queens (Guidugli *et al.,* 2005) and is also expressed in the fat body and ovaries of non-reproductive worker bees (Cardoso-Junior *et al.,* 2021).

Molecular data suggest that exposure to imidacloprid alters the expression of genes associated with development, detoxification, oxidation-reduction, and immunological response in honey bees. Oral exposure to imidacloprid altered the expression of vitellogenin and genes involved in memory formation in the brain of honeybees (Christen *et al.,* 2016). Imidacloprid altered the mRNA expression levels of the P450 superfamily, genes involved in the coding of antioxidant enzymes (catalase, superoxide dismutase, and thioredoxin peroxidase), and immunity (apidaecin) (Chaimanee *et al.,* 2016). The mRNA expression levels of genes involved in ribosomal protein activities, phototransduction, visual perception, and photoreception, actin binding, and somatic muscle development were altered by exposure to imidacloprid (Wu *et al.,* 2017). Imidacloprid has adverse effects on the ecological sustainability of honey bees, as chronic pesticide exposure can damage an entire colony or population and even pass on to future generations (Chen *et al.,* 2021).

1.6. The detoxification system

During their lives, honey bees are exposed to a variety of stressful environmental conditions, including pesticides that generate reactive oxygen species (ROS) and lead to oxidative damage to cellular biomolecules (Zhu *et al.,* 2016). Oxidative stress refers to an imbalance in the redox status of the body due to an overproduction of free radicals that exceeds the ability of the antioxidant defense system to neutralize them (Birben *et al.,*

2012). Uncontrolled free radical production can pose a serious threat to the survival of body tissues by damaging vital cellular components such as membranes, lipids, nucleic acids, and proteins (Hodgson and Smart, 2001). Elevated ROS can lead to DNA mutations (Imlay and Linn, 1988), loss of enzyme activity and lipid peroxidation (Stadtman and Levine, 2003), all of which affect normal honey bee behavior. Aerobic organisms are equipped with protective mechanisms that include enzymatic and non-enzymatic antioxidants, which are normally effective in blocking the harmful effects of free radicals, including ROS (Birben *et al.,* 2012).

The midgut of the honey bee is the first route of entry for pathogens and toxic substances (Catae *et al.,* 2014). It is more sensitive to toxic substances (Bielenin and Ibek, 1980) and malnutrition (Szymas and Przybyl, 2007) due to the absence of chitinous lining. In the midgut, where all substance absorption occurs, insecticide-induced morphological changes can affect various physiological and behavioral functions (Williamson *et al.,* 2014; Catae *et al.,* 2014). The peritrophic membrane, an anatomical structure surrounding food, plays an important role in insect gut biology. It protects the midgut epithelium from mechanical damage and from toxic substances (Bielenin and Ibek, 1980). The PM can act as a semipermeable membrane that divides the midgut lumen into distinct, physiologically relevant compartments and regulates the transit of molecules between midgut compartments (Lehane, 1997).

Metabolic resistance is one of the most important mechanisms used by insects to escape the harmful effects of natural and synthetic toxins (Rand *et al.,* 2015). Honey bees have endogenous antioxidant enzymes and a detoxification system to protect themselves from natural and synthetic toxins. Catalase (CAT), peroxidases (POD), superoxide dismutase (SOD), glutathione S-transferase (GST), and glutathione peroxidases (GPx) are involved in the antioxidant mechanisms of honeybees (Weirich *et al.,* 2002). The antioxidant enzymes in honeybees are efficient in detoxification of ROS and SOD act as the first line of defense against oxygen free radicals (Corona and Robinson, 2006). H_2O_2 is eliminated by the action of CAT (Aebi, 1984) and glutathione peroxidases (Mannervik, 1985). The GST conjugate the toxic compounds to glutathione to facilitate their removal from the organism (Hinton *et al.,* 1995; Jakoby, 1985; Lee, 1991). Because honeybees have a lower number of genes coding for antioxidant proteins in their genome, the ability

of the honey bee to defend itself against ROS is strongly limited compared to other insects (Corona and Robinson, 2006).

Although there are anatomical structures for nonspecific defense against toxins, various biochemical and molecular mechanisms also play important roles in the defense against toxins. Cytochrome P450 monooxygenases, glutathione transferases, and carboxylesterases are the major enzyme families involved in detoxification of natural and synthetic toxins into less toxic substances (du Rand *et al.,* 2017). Cytochrome P450 enzyme superfamilies are widely distributed multifunctional enzymes belonging to four clades involved in environmental responses and detoxification. AccCYP336A1 is a member of the P450 family that belongs to the CYP3 clade and has a high degree of homology with Am CYP336A1 of *Apis mellifera* and is involved in the response to various oxidative stresses (Zhu *et al.,* 2016).

The number of protein-coding genes in the honeybee genome has greatly reduced, especially in three superfamilies that code for enzymes that detoxify xenobiotics. The number of glutathione S-transferases, cholinesterases and cytochrome P450 monooxygenases (P450s) in honeybees is specifically less than half that of other insects (Claudianos *et al.,* 2006). Several clades in these superfamilies contain genes that have been specifically linked to the detoxification of xenobiotics. These three superfamilies play a significant role in pesticide metabolism in other insects, and they are responsible for the vast majority of mutations resulting in metabolic resistance to chemical insecticides (Feyereisen, 2012).

1.7. Nutrition and Ascorbic acid supplementation

Proteins (amino acids), carbohydrates (sugars), lipids (fatty acids, sterols), vitamins, minerals (salts) and water are necessary for the nutrition of honey bees. Healthy development of the bee colony is supported by consumption of these nutrients in a certain qualitative and quantitative ratio (Brodschneider and Crailsheim, 2010). The most important protein and carbohydrate sources in the natural diet of the honey bee are pollen and nectar, respectively. Ascorbic acid, biotin, vitamins D and E, folic acid, mositol, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine are among the vitamins found in pollen (Standifer, 1980; Keller *et al.,* 2005). Unlike fat-soluble vitamins, water-soluble vitamins (such as vitamin C) are commonly found in pollen;

however, their amounts may vary depending on the season and flower source (Roulston and Cane, 2000).

For nurse bees to raise their brood, they need the vitamins thiamine, ascorbic acid, riboflavin, nicotinamide (niacin, nicotinic acid), pyridoxine, pantothenic acid, folic acid, and biotin. Pantothenic acid and nicotinic acid are required for differentiation between workers and queens (Trager, 1947; Somerville, 2000). Royal jelly is composed of a variety of elements, including fats, proteins, and carbohydrates, as well as smaller amounts of vitamins, minerals, and phenolic or volatile chemicals. When bees begin producing royal jelly for the growing larvae and queen, they require a diet rich in vitamins (Collazo *et al.,* 2021).

Supplemental feeding can help the colony survive or increase its population and productivity when nectar and pollen are limited. Supplemental feeding with vitamins and probiotics can increase body weight and life expectancy (Collazo *et al.,* 2021). Ascorbic acid appears to be necessary for brood rearing in honey bees, and vitamin C levels correlate strongly with the stage of larval development (Sawczuk *et al.,* 2022). Honey bees are thought to be able to synthesize vitamin C (Ulutas and Ozkirim, 2018). It is synthesized either from simple precursors or from symbiotic microorganisms in the gut of honeybees (Herbert *et al.,* 1985).

Ascorbic acid or vitamin C (C6H8O6) is an important water-soluble antioxidant that can effectively neutralize free radical-containing reactive oxygen species (ROS), thereby protecting cells from oxidative damage such as peroxidation-mediated damage to biomolecules (Verma *et al.,* 2007). It acts as a cofactor for several types of metabolic enzymes. It can act both inside and outside cells to combat free radical damage and act as an electron source for free radicals such as hydroxyl and superoxide radicals and suppress their reactivity (Bendich, 1990, Bindhumol *et al.,* 2003). It has been shown to be a useful antioxidant in preventing lipid peroxidation in cell membranes, especially those surrounding and within intracellular organelles (Bendich *et al.,* 1986).

It is commonly used in everyday life as an ingredient in foods and dietary supplements. It provides a variety of benefits, including those related to detoxification, immune system boosting, anti-aging, antiviral and antibacterial activities (Kramer and Seib, 1982). It is a unique antioxidant because it contributes only one reducing equivalent

and the radical it forms, monodehydroascorbate, reacts preferentially with other radicals rather than with nonradical substances (Njus *et al.,* 2020). Ascorbic acid is a superior reducing agent, generating the ascorbate radical after two successive oxidations. Due to the presence of a stable unpaired electron in ascorbic acid, it is often non-reactive. The enzyme structure is maintained by the use of reducing agents, which allows the biochemical machinery of cells and tissues to function normally (Zhitkovich, 2021). Dietary supplementation with ascorbic acid has a positive effect on antioxidant defense and thus on winter mortality in honey bees (Farjan *et al.,* 2012).

Insects have a high metabolic rate, which naturally generates large amounts of free radicals. Any type of stress, such as exposure to pesticides, can also stimulate the production of ROS. Therefore, an efficient antioxidant system is of particular importance for insects. To prevent the undesirable ROS effects, organisms develop defense mechanisms that include both enzymatic and non-enzymatic components. The main antioxidant enzymes in insects are superoxide dismutase, catalase, peroxidases, etc. The ability of honey bee to defend itself against ROS appears to be severely limited compared to other insects because there are fewer genes in the genome those codes for antioxidant proteins. Antioxidants such as ascorbic acid are involved in minimizing oxidative damage to proteins, DNA/RNA, and other important bio macromolecules. However, the interaction between the protective effects of antioxidants and the elevated levels of ROS, which occur when honey bees are exposed to imidacloprid, is still unknown.

The present study proposes to analyze the stress and associated biochemical changes produced by exposure to imidacloprid and to investigate the effects of ascorbic acid supplementation on peroxidative damage and defense mechanisms. It also proposes to study the effect of ascorbic acid supplementation on reproductive genotoxicity of honey bees exposed to imidacloprid by mRNA expression studies.

The objectives of the thesis include the following:

The effect of imidacloprid and supplementation of ascorbic acid on

I. The biochemical changes associated with the midgut tissues and hemolymph in worker bees.

II. The changes associated with antioxidative system.

III. The differential expression of genes associated with caste formation and reproductive status in queen bees.

IV. The histological alterations associated with the reproductive structures in queen bees.

Chapter 2.
Review of Literature

Contents

Chapter 2. Review of Literature

The widespread use of systemic neonicotinoid pesticides is one of the factors responsible for the decline in honey bee population (Goulson *et al.,* 2015). Compared to other neonicotinoids, imidacloprid is known to alter the physiology and health of honey bees at relatively low concentrations (Codling *et al.,* 2016). They are neurotoxins that act as agonists of the nicotinic acetylcholine receptor (Christen *et al.,* 2016). Exposure to imidacloprid affects metabolic pathways, the antioxidant system, and also histological architecture, which may affect colony health and productivity (Paleolog *et al.,* 2021; Carneiro *et al.,* 2022). Exposure to imidacloprid has negative effects on the reproductive capacity of honey bee queens, which can negatively impact colony performance and survival (Kozii *et al.,* 2021; Moreira *et al.,* 2022). It is imperative to implement conservation and management techniques to counteract honey bee population declines and maintain global ecosystem services and economic benefits.

The present study addresses the effect of ascorbic acid supplementation on biochemical parameters, antioxidant system, gene expression levels, and histological changes in worker bees and queen bees after exposure to imidacloprid. A review of the effects of neonicotinoids on the morphological, physiological, biochemical, and reproductive characteristics of honey bees that affect honey bee health and survival was conducted. The study critically analyzed the sublethal effects of neonicotinoids on nAChRs, antioxidant system, reproduction and behavior. In addition, the role of ascorbic acid supplementation in honeybees to enhance antioxidant activities and detoxification system against imidacloprid toxicity was investigated.

2.1. Honey bee decline

Numerous interacting factors have been linked to bee colony declines (Appenfeller *et al.,* 2020). Wild pollinator declines and honey bee colony losses are caused by constant exposure to numerous interrelated stressors. Bees are subjected to severe stress from parasites, pesticides, and flower scarcity. Exposure to pesticides can impair detoxification

processes and immunological responses, making them more susceptible to parasite infestations (Goulson *et al.,* 2015).

Gallai *et al.,* (2009) demonstrated the economic importance of insect pollinators to the global consequences of pollinator decline by calculating the contribution of insect pollination to the economic value of global agricultural production and the vulnerability of global agriculture to pollinator decline.

Colony Collapse Disorder (CCD), a special case of collapse or rapid loss of adult honey bees, resulted in significant economic loss to farmers. (Brutscher *et al.,*2016; Dively *et al.,* 2015). The first systematic study of bee populations affected by Colony Collapse Disorder was conducted by VanEngelsdorp *et al.,* (2009), who hypothesized that CCD is caused by a combination of diseases and other stressors. Classification and regression tree analysis showed that CCD is most likely the result of numerous factors interacting to make infected colonies more susceptible to disease (VanEngelsdorp *et al.,* 2010).

O'Neal *et al.,* (2018) found that a number of factors, including exposure to parasites and pathogens, habitat loss, reduced availability or quality of food resources, climate change, poor queen quality, development of commercial and cultural beekeeping practices, and exposure to agricultural and apicultural pesticides both in the field and in the hive, have negative effects on honey bee colony health and survival.

Harwood and Dolezal, (2020) studied the combined effects of viruses and pesticide exposure negatively impacting the health of honey bees. The interactions between pesticides and viruses affect honey bee colonies at the individual and colony level due to the complicated social structure of honey bee colonies. Dicks *et al.,* (2021) reported the importance of pollinator loss at regional and global levels and its impact on human welfare. They considered that pesticide use and land management are the most important factors for pollinator decline.

Xiao *et al.,* (2022) conducted a study to track pesticide exposure in hives through oral ingestion of contaminated hive matrices. The observations showed that pesticides were found in 93.6 percent of pollen, 81.5 percent of nectar, 96.6 percent of bee bread, and 49.3 percent

of honey. These empirical results contribute to the understanding of the extent at which agricultural pesticide contaminated honey bee hives and their products.

Indhu *et al.,* (2022) conducted a survey of global pollinator decline. They focused on *Apis cerana indica* F., as this species is critical for maintaining ecological balance and transforming current agricultural landscapes into agroecosystems in tropical peninsular India. They discussed the need for long-term studies of plant-pollinator networks to manage and conserve native Asian honeybees.

Theisen-Jones and Bienefeld, (2016) conducted a survey across Asia to investigate the factors contributing to the decline of *A. cerana* populations. According to them, numerous direct and indirect factors, such as rapid conversion of forests to rubber and oil palm plantations, short-cycled forest stands, and other agricultural operations, contribute to the decline of *A. cerana* populations. Liu *et al.,* (2022) analyzed the presence of environmental pollutants in *A. cerana* to investigate their toxicity and survival risk. They detected several pesticide residues in *A. cerana*, including neonictinoids.

2.2. The impact of Neonicotinoids

Kurupke *et al.,* (2012) established multiple pesticide exposure routes at harmful levels in honey bees residing close to agricultural fields. Multiple pesticides were found in pollen collected by honey bees and pollen collected from treated plants, including neonicotinoids, atrazine, and fungicides. Neonicotinoid pesticides have been detected in soil, dust, planter exhaust, water (guttation) droplets exuded by treated and untreated plants and the nearby fields. The pollen contaminated with neonicotinoid and collected by the worker bees is concerning, as the developing bees are exposed to these pesticides through the stored pollen in the hive.

Neonicotinoids have altered the agrochemical environment for pollinators and have a wide range of harmful sub lethal effects on honeybees at field realistic levels. Other agrochemicals can increase the toxicity of neonicotinoids, and they work in concert with infectious pathogens like *Nosema ceranae* to cause colony collapse (Van der Sluijs *et al.,* 2013).

Carillo *et al.,* (2013) investigated the effects of imidacloprid and fipronil on *Apis mellifera* L. learning behaviour. They discovered that the insecticides imidacloprid and fipronil impaired bee learning and are particularly detrimental to foraging. Oral imidacloprid treatment of honeybees decreased medium-term olfactory memory and proboscis extension reflex, but the short-term and long-term memories were unaffected. The effects of imidacloprid on medium-term olfactory memory are examined in the context of neuronal circuits in the honeybee brain that are thought to mediate memory formation (Decourtye *et al.,* 2004).

Tan *et al.,* (2014) demonstrated that the sublethal dosages of imidacloprid can impair honey bee decision-making by raising the probability of a bee approaching a potentially hazardous food source. They found that sublethal quantities of the imidacloprid, which is extensively used in Asia, can inhibit the foraging of *A. cerana*.

Codling *et al.,* (2016) detected the presence of Neonicotinoid insecticides and their transformation products in honey, pollen and in honey bees. Imidaclorpid, imidaclorpid-Olefin, and imidacloprid-5- Hydroxy transformation products were detected more frequently and at higher mean concentrations. Clothianidin and thiamethoxam were also found in honey samples, pollen samples and in bees. The potential dietary uptake of these neonicotinoids from honey and pollen by bees might have negative effects on the proper functioning of the colony.

Wilde *et al.,* (2016) studied the effect of sublethal doses of imidacloprid on protein content and activity of proteases on honey bees. Their results showed a decrease in protein biosynthesis and a reduction in the level of proteolytic activity in *A. mellifera.*

Wessler *et al.,* (2016) found that, when the honey bee colonies were exposed to nenicotinoids, the acetylcholine release from the hypopharyngeal gland was reduced. The secretory vesicle in the hypopharyngeal gland was found to be damaged. Their results suggest that, a reduction of the ACh content in the brood food on exposure to the neonicotinoids may adversely affect brood development and loss of honeybee colonies. Heylen *et al.,* (2011) studied the impact of sublethal doses of 4 pesticides on the size and morphology of the hypopharyngeal glands of honeybee workers. The size of the acini was significantly reduced in the bees exposed to imidacloprid. Hatjina *et al.,* (2013) investigated

the sublethal effects of imidacloprid on the development of the hypopharyngeal glands in honeybees under laboratory conditions. The size of the acini was found to be reduced in the hypopharyngeal glands of imidacloprid-treated honeybees.

The global transcriptome patterns in the brain of honey bee workers exposed to environmentally realistic and sublethal concentrations of imidacloprid showed considerable changes in gene expression, which were often concentration-dependent. They were able to modify the expression of several metabolic pathways as well as confirm the down-regulation of major royal jelly proteins (Christen *et al.,* 2018)

Colin *et al.,* (2019) fitted bees with RFID tags to monitor their lifetime flight behavior. Bees started foraging earlier as adults and made fewer orientation flights as a result of the imidacloprid residues, which also resulted in a 28% decrease in lifetime foraging flights. They asserted that imidacloprid might have an impact on colony function by unbalancing the colony's typical age-based division of labour and decreasing foraging effectiveness. Cook (2019) studied the effect of chronic oral exposure of honey bees to two sub lethal concentrations of clothianidin and imidacloprid. Neonicotinoids altered important aspects of honey bee nutritional and metabolic physiology in a compound and dose-dependent manner and interfered with honey bee endocrine neurophysiological pathways.

Grunewald and Siefert, (2019) conducted a review on the effect of neonicotinoids on honey bees. They suggest that neonicotinoids pose risks to pollinating bees that include neuronal effects that affect the behavior of mature individuals and non-neuronal effects that disrupt development and glandular function. The neonicotinoid exposure may result in the decreased secretion of ACh for brood feeding and the shrinkage of the hypopharyngeal gland in turn adversely affect the colony.

Delkash-Roudsari *et al.,* (2020) observed that chronic exposure to glyphosate may reduce pollination success. The acute exposure to imidacloprid and ethion negatively affect honey bee foragers, with the effects of chronic exposure being dose-dependent for these two insecticides. Vinothkumar *et al.,* (2020) performed bioassay studies to determine the median lethal concentration values and safety indices for *A. cerana indica* on exposure to neonicotinoids. The least safe chemicals for bees, according to the safety index, are thiamethoxam and imidacloprid.

Pisa *et al.,* (2021) reviewed the impact of systemic insecticides including neonicotinoids on the ecosystem services and functioning, particularly on pollination, soil biota, and aquatic invertebrate communities. According to them, high toxicity of these systemic insecticides to invertebrates (insects and crustaceans) which have an ecological impact on pollinators has been confirmed. They have sub-lethal effects on fish, frogs, reptiles, birds, and mammals, demonstrating a better understanding of the mechanisms underlying the toxicity of these insecticides in vertebrates and their harmful effects on the development, reproduction, and neurobehavioral functions.

Chen *et al.,* (2021) conducted a review on the effects of imidacloprid on the honey bees. The lethal dose/concentration of imidacloprid in the honey bee *Apis mellifera* and molecular effects of sub lethal dosages of imidacloprid were integrated and reviewed. The levels of lethal dose/concentration in honey bees *Apis mellifera*, fluctuate with the seasons and geographical locations. The expression of genes related to the immune system, detoxification, oxidation-reduction and other aspects of development was affected in honey bees on exposure to sub lethal doses of imidacloprid.

After acute oral exposure, Carneiro *et al.,* (2022) assessed the toxicity, histopathology, cytotoxicity, and expression of the autophagy-related gene atg1 in the midgut of *A. mellifera.* Midgut epithelium of bees exposed to imidacloprid exhibits cytoplasm vacuoles, increased intercellular gaps, striated border disorganisation, nuclear pyknosis, and an organ injury index that rises with exposure time. They claim that high levels of cell death in the midguts of workers exposed to imidacloprid may adversely affect the ability to digest food.

Sowmiya *et al.,* (2022) studied the foraging behavior of *Apis cerana indica.* According to the results of their study, neonicotinoids are increasingly preferred by Indian bees, particularly nitro-substituted neonicotinoids like imidacloprid and clothianidin, which raises the danger of pesticide exposure on the bee colony.

2.3. Neonicotinoids as agonists of nAChRs

Mastuda *et al.,* (2001) investigated the toxicity of imidacloprid on nAChRs and their results suggest that α- and non-α-subunits of nAChRs contribute to the interaction of these receptors with imidacloprid. According to their studies in binding assays, molecular biology,

and electrophysiology, the selective toxicity of imidacloprid could be caused by electrostatic interactions between its nitroimine group and the bridgehead nitrogen with specific nAChR amino acid residues. Brown *et al.,* (2006) reported the effects of acetylcholine (ACh), nicotine, and the neonicotinoids on native nAChRs in cholinergic neurons grown from *Drosophila* larvae. Their results showed that neonicotinoids can have a variety of effects (antagonist, partial agonist, or superagonist) on nAChRs.

Christen *et al.,* (2016) studied the molecular effects of four neonicotinoids (imidacloprid, acetamiprid, clothianidin, and thiomethoxam) and nicotine on genes associated with nicotinic acetylcholine receptor units, the multifunctional gene vitellogenin, and genes associated with the immune system. They examined the transcriptional changes of eight selected genes in the brains of honeybees after exposure for 24, 48, and 72 hours. According to their observations, nAChR transcripts were upregulated upon exposure to nicotine and neonicotinoids. The upregulation induced by neonicotinoids and nicotine may be due to the loss of function of these receptors. In addition to effects on nAChRs, neonicotinoids induced transcriptional changes in genes related to immunity and brain function.

Christen *et al.,* (2017) determined the transcriptional changes of nAChRs (nAChRα1 and nAChRα2) and vitellogenin in the brain of honey bees *Apis mellifera* after exposure to neonicotinoids over a 72-hour period. Neonicotinoids induced changes in gene transcripts associated with the immune system and genes related to memory formation, with negative effects on the immune system, foraging, and memory formation in worker bees. The affinity of nAChRs is related to the type and strength of neonicotinoid used. According to the studies by Tailebois *et al.,* (2018), the affinity of neonicotinoids for imidacloprid binding sites in insects varies with the type of neonicotinoid as well as the insect species exposed.

Acetylcholine is an important signaling substance in both individual bees and honey bee colonies. It is critical for colony formation and vitality because it acts on neuronal and presumably non-neuronal pathways and is socially transmitted between individuals. Insecticides that interfere with these signaling molecules are disrupted and cause unanticipated side effects, even when used at low concentrations (Grunewald and Siefert, 2019). Boily *et al*., (2013) investigated the effects of imidacloprid exposure on AChE

activity. Their results showed that imidacloprid caused an increase in AChE activity, which can be used as a possible biomarker for neonicotinoid exposure.

Cabirol and Haase, (2019) described the action of neonicotinoids as agonists of nicotinic acetylcholine receptors and the structural and functional changes in the brain responsible for behavioral changes in honey bees. The affinity of neonicotinoids for these receptors depends on the structure of nAChRs and the location of the receptor in the brain. Imidacloprid shows different binding properties to nAChR subtypes in different species. Neuronal responses to neonicotinoids may vary depending on the age and developmental stage of honey bees. Increased expression of nAChR subtypes upon oral exposure of bees to neonicotinoids may result in increased olfactory stimulation and altered flight behavior.

Shigetou *et al.,* (2020) investigated the agonist activity of imidacloprid, thiacloprid, and clothianidin for the two hybrid nAChRs and found that imidacloprid showed higher affinity and lower potency than clothianidin for the nAChRs. Thiacloprid showed the highest agonist affinity and lowest efficacy among the three neonicotinoids studied.

The effects of neonicotinoids on the mammalian nervous system were studied by Costas-Ferreira and Faro, (2021). They claimed that neonicotinoids cause neurobehavioral toxicity due to their modulatory effects on nAChRs, which can lead to neurochemical changes such as decreased nAChR expression and alterations in acetylcholinesterase activity. All of these consequences have the potential to activate various intracellular signaling pathways, which in turn cause oxidative stress, neuroinflammation, and ultimately neuronal death.

2.4. Effect on reproduction

Exposure to neonicotinoids has negative impacts on the colony performance of honey bees. It results in reduction in the number of adult bees, honey production, and pollen collection. Within one year of neonicotinoid exposure, colonies showed signs of queen failure and queen supersedure (Sandrock *et al.,* 2014). Exposure to field-realistic concentrations of neonicotinoid pesticides resulted in reduced queen success, altered reproductive anatomy (ovaries) and physiology (quality and quantity of sperm stored in the spermatheca) in the queens of *A. mellifera* (Williams *et al.,* 2015).

Wu-Smart and Spivak, (2016) studied the effects of imidacloprid on the fecundity of queen bees by administering different concentrations of imidacloprid in small colonies of different sizes. They found that imidacloprid affected queen bee oviposition and locomotor activity of queen bees, behavior of worker bees and colony development in all imidacloprid-treated colonies. The results also showed that queens in small colonies were more susceptible to the exposure.

The effects of coumaphos and imidacloprid on the viability of sperm retained in the spermatheca of honey bee queens were studied by Chaimanee *et al.,* (2016). Their results showed that sublethal doses of imidacloprid reduced sperm viability. Honey bee queens treated with low doses of coumaphos (5 ppm) and imidacloprid had reduced expression of CYP306A1, CYP4G11, and CYP6AS14 (0.02 ppm). In addition, these two chemicals inhibited the expression of antioxidant, immunity, and developmental genes in the queens.

Brandt *et al.,* (2017) investigated the immunocompetence of *Apis mellifera* queens. Queens exposed to neonicotinoids had significantly fewer hemocytes and a lower proportion of active, differentiated hemocytes. Their results indicated that neonicotinoid insecticides can adversely affect the immunocompetence of queens, resulting in a lower potential for disease resistance.

Imidacloprid exposure has adversely effects on survival, growth, physiology and immunity of honey bee workers. Vitellogenin (Vg) may be a suitable biomarker for evaluating energy stress and sublethal effects of pesticides on honey bees. Imidacloprid exposure could lead to increased energy expenditure and reduction in vitellogenin levels in honey bees (Abbo *et al.,* 2017). Christen *et al.,* (2019) evaluated vitellogenin expression in brain, fat body, and hemolymph after exposing bees to the neonicotinoid, clothianidin for 24 hours. They produced a rabbit polyclonal vitellogenin antibody to evaluate the changes at the protein level. Accordingly, vitellogenin can be used as a potential biomarker for bee exposure to neonicotinoids and other pesticides, since an increase in vitellogenin was observed in response to clothianidin exposure. Vergara-Amado *et al.,* (2020) studied the effect of imidacloprid and the reproductive status of queen bees on their energy metabolism. They found a decrease in the standard metabolic rate in queens exposed to imidacloprid. Their results suggest that the reduction in metabolic rate may affect queen health and colony performance.

Neonicotinoids have a deleterious impact on the larval survival, reproductive capacity, and histopathology of honey bee queens. Exposure to the neonicotinoid reduced larval and pupal survival and sperm viability in mated queens. These changes have an impact on queen development and reproductive longevity (Kozii *et al*., 2021). Moreira *et al.,* (2022) studied the effects of imidacloprid in the midgut and ovaries of *Apis mellifera* queens. Imidacloprid exposure showed changes in the external musculature and cellular changes in the midgut of queens. The digestive cells in the midgut showed pyknotic nuclei, indicating a cell death process. Degeneration and resorption of ovariole contents were the predominant changes in the ovaries of these reproductive bees treated with imidacloprid, probably negatively affecting colony growth and fertilization.

2.5. Effect on antioxidative system

Korayem *et al*., (2012) studied the protective strategy of antioxidant enzymes against hydrogen peroxide in honeybees in two different seasons. They found that when the total peroxide level increased, the activities of superoxide dismutase and ascorbate peroxidase were also increased. According to their findings, antioxidant enzymes keep the redox state of the cell stable and prevent free radicals from causing damage that would affect normal cellular functions.

Badawy *et al.,* (2015) examined the effects of four different insecticides on honey bees to investigate the potential mechanism of action of the insecticides as well as detoxification of honey bees and resistance to these insecticides. The specific activities of acetylcholinesterase, carboxylesterase, glutathione S transferase, and polyphenol oxidase varied in different tissues of honeybees after exposure to these insecticides. Their results suggest that enzyme activity may serve as a reliable biomarker for exposure to agrochemicals.

Zhiguo Li *et al.,* (2017) investigated the effect of imidacloprid on the expression of immune related genes and the activities of carboxylesterase, prophenoloxidase, acetylcholinesterase, and glutathione S transferase in *A. cerana* and *A. mellifera*. The activities of carboxylesterase, prophenoloxidase, and acetylcholinesterase were found to be upregulated in *A. cerana* upon exposure to imidacloprid. Their study suggests that *A. cerana* and *A. mellifera* exhibit different patterns of immune related gene expression and enzymatic

activities upon exposure to imidacloprid. Exposure to imidacloprid decreased the lifespan, food intake, and antioxidant gene expression in honey bees. It induced down-regulation of antioxidant genes and caused severe harm to the tissues of the midgut (Gregorc *et al.,* 2018).

Pizzaia *et al.,* (2020) compared the enzymatic activity and total antioxidant capacity of worker bees of *Apis mellifera* tolerant to thiomethoxam with those of non-tolerant worker bees. Compared to the non-tolerant group, the results showed that the tolerant bees had a significant difference in enzymatic activity and total antioxidant capacity to reduce the damage caused by thiamethoxam. Paleolog *et al*., (2021) studied the antioxidant defenses in workers and queen bees of *Apis mellifera* after exposure to imidacloprid. They determined the activities of CAT, GPx, GST, and SOD, as well as the level of total antioxidant potential in hemolymph. Imidaclopid suppressed the antioxidant defenses of the workers. However, antioxidant defenses of queens were found to be increased upon exposure to imidacloprid.

Murawska *et al.,* (2021) reviewed the biochemical effects of neonicotinoids in honey bees, with special emphasis on the effects on cytochrome P450 monooxygenases, glutathione transferases, and carboxylesterases. They also investigated the effect of neonicotinoids on the activities of catalase, superoxide dismutase, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase. Accordingly, neonicotinoids can cause biochemical changes that affect the condition, health, and performance of bee colonies.

The effects of imidacloprid on body weight, flight performance, carbohydrate content and lipid content of forager bees were studied by Kim *et al.,* (2022). It caused down-regulation of genes involved in carbohydrate and lipid metabolism in forager bees. Body weight and flight ability of worker honeybees were found to decrease in a concentration-dependent manner. The results suggest that imidacloprid in practical doses alters the energy metabolism of honey bees, which is detrimental to the health of honey bee colonies.

2.6. Oxidative stress and detoxification

The number of genes encoding the carboxyl/cholinesterases (CCE), cytochrome P450 monooxygenases (P450), and glutathione S transferases superfamilies is significantly lower in the honeybee genome. These deficiencies in xenobiotic detoxification enzymes contribute to the sensitivity of honeybees to xenobiotics (Claudianos *et al.,* 2006).

Zhu *et al.,* (2016) isolated P450 and characterized AccCYP336A1 gene from *A.cerana cerana* and investigated its role in resistance to oxidative stress. They showed the expression patterns of AccCYP336A1 at mRNA and protein levels in response to oxidative stress at different developmental stages. AccCYP336A1 showed the highest expression level at the first larval stage and the lowest at the adult stage. Its tissue-specific expression pattern showed higher expression in the epidermis. Real-time quantitative PCR analysis showed an increase in the expression of AccCYP336A1 when honeybees were exposed to 4ºC, 42ºC, UV radiation and H_2O_2. AccCYP336A1 expression was induced by the pesticides thiomethoxam, methomyl, deltamethrin, and phoxim. Accordingly, the induction of the gene CYP is a response to various oxidative stress factors. These results support the role of the gene in protection against oxidative stress.

De Smet *et al.,* (2017) examined the expression levels of stress-related genes upon exposure to imidacloprid. Increased levels of the detoxification genes CYP9Q3 and CYT P450 were observed when honeybees were exposed to imidacloprid. Hypopharyngeal gland size was found to decrease when exposed to imidacloprid. When honey bees were treated with imidacloprid in the laboratory, they showed general immunosuppression and lack of detoxification mechanisms, but when exposed in the field, they showed a resistant response with immune activation over time.

To better understand the detoxification mechanism, du Rand *et al.,* (2017) conducted an experiment to detect the proteomic and metabolomic responses of nicotine-fed larvae. They found conclusive evidence that larvae are able to effectively metabolize a dietary toxin, implying that the sensitivity of larvae to certain toxins is not due to a decreased detoxification capacity. The results showed that larvae are able to tolerate and reject hazardous substances such as pesticides present in the environment.

The antioxidant effect of caffeine against oxidative stress caused by imidacloprid was investigated by Balieira *et al.,* (2018). Both catalase and glutathione peroxidase activities were increased by imidacloprid. The two imidacloprid-fed groups showed a significant decrease in the amount of proteins containing the thiol group, whereas the addition of caffeine abolished the adverse effect caused by imidacloprid. Malondialdehyde concentration was increased by imidacloprid, although this effect was substantially reversed by the addition of caffeine. They claimed that imidacloprid caused oxidative damage by increasing food

intake, which was partially attenuated by caffeine. The results indicated that the sublethal effects of this insecticide on honey bees can be reduced by caffeine treatments.

Wang *et al.,* (2018) reviewed the relationship between neonicotinoid toxicity and metabolism and the formation of reactive oxygen species, reactive nitrogen species, and oxidative stress as a result of neonicotinoid treatments. They emphasized the significant roles of oxidative stress in neonicotinoid-induced toxicity to non-target species. Shi *et al.,* (2018) conducted a study to understand the complex interactions between neonicotinoids and honey bees. Honey bees have activated their detoxification system to resist oxidative stress induced by neonicotinoids.

Using a MALDI imaging method, Catae *et al.,* (2019) examined the effects of exposure to a sublethal dose of imidacloprid on the distribution of proteins in the brains of *Apis mellifera* worker bees. The results suggest that imidacloprid treatment resulted in a number of biochemical changes, including alterations in synapse modulation, apoptotic regulation, and oxidative stress, which may adversely affect bee physiology.

To elucidate the effects of imidacloprid on honeybee learning ability, a comparative RNA-Seq transcriptome analysis was performed by Li *et al.,* (2019). The olfactory learning of bees was severely affected by prolonged exposure to imidacloprid. It was found that 130 genes in the brain transcriptome of imidacloprid-treated bees were down-regulated. According to gene ontology annotation, oxidative stress induced by imidacloprid exposure may have caused an imbalance between oxidation and reduction in brain tissue. Their results suggest that the lower olfactory learning ability in imidacloprid-treated bees could be caused by the downregulation of genes involved in immunological, detoxification, and chemosensory responses.

Almasri *et al*., (2020) studied the effect of imidacloprid on physiological markers involved in the nervous system, detoxification, oxidative stress, metabolism, and immunity. Acetylcholinesterase in the head, glucose-6-phosphate dehydrogenase in the abdomen, glutathione S-transferase, and midgut alkaline phosphatase in the midgut showed modulations upon exposure to imidacloprid. To investigate the sublethal toxicity of neonicotinoids in Asian honeybees, Gao *et al.,* (2020) analyzed the expression patterns of differentially expressed genes (DEGs) by trend analysis and performed gene ontology analysis and KEGG pathway enrichment analysis. Enzyme activities of P450 and Carboxyl

esterase were reduced when bees were exposed to imidacloprid, but GST activity was not affected by pesticide exposure. They found that genes associated with metabolism, catalytic activity, and structural molecule activities were downregulated.

He *et al.,* (2021) investigated the toxicity of imidacloprid by administering sublethal and median lethal doses of imidacloprid. Their results showed that reactive oxygen species (ROS), Fe2+ and mortality rate of bees were increased in a dose-dependent manner by imidacloprid exposure. The ferritin gene (AmFth), which is essential for attenuating Fe2+ overload, was found to be upregulated upon exposure to imidacloprid. Therefore, they claimed that activation of ROS, which is induced by iron overload, is the cause of imidacloprid toxicity.

Kumar *et al.,* (2022) evaluated the molecular tissue damage focusing on apoptosis in honeybees. According to their results, there was an increase in both mitochondria- and endoplasmic reticulum (ER) -mediated apoptosis in the body tissues of honeybees, which is supported by significant oxidative stress and leads to programmed cell death. Tissue homogenates from bees exposed to pesticides showed increased levels of reactive oxygen species and activation of caspase3.

Pal *et al.,* (2022) investigated the effects of the insecticide imidacloprid on oxidative stress by measuring the activity of superoxide dismutase, catalase, glutathione S-transferase, glutathione reductase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase in the head, abdomen, and midgut. According to their findings, imidacloprid caused oxidative stress in honeybees, which may be responsible for much of the toxicity of imidacloprid to honeybees.

2.7. Ascorbic acid supplementation

Ascorbic acid plays an important role as an antioxidant element of plasma, extracellular fluids surrounding the lung, lens, and retina, and protection of phagocytic cells involved in pathogen invasion (Bendich *et al.,* 1986).

Farjan *et al.,* (2012) investigated the effects of vitamin C (ascorbic acid) supplementation on glutathione content, total antioxidant status (TAS) and activity of antioxidant enzymes in bee brood, including superoxide dismutase, peroxidase and catalase and glutathione transferase. Their results showed a significant increase in protein and

glutathione levels, as well as an increase in the activities of peroxidase, catalase and glutathione transferase in workers emerging from bee colonies after receiving vitamin C supplements. The impact of vitamin C on the antioxidant system and antioxidative enzyme activities in newly emerged workers of *Apis mellifera carnica* colonies naturally parasitized with *Varroa destructor* was examined by Farjan *et al.,* (2014). Their results showed that the activities of antioxidative enzymes were enhanced on supplementation with vitamin C.

Andi and Ahmadi, (2014) studied the effect of vitamin C supplementation on rate of egg laying by the queen, colony number, body weight, and protein content in *Apis mellifera*. The results showed that vitamin C supplementation increased brood area, colony population, and body weight and protein content of workers.

Farjan *et al.,* (2015) studied the effect of supplementation of vitamin C on carbohydrate metabolism in the developing brood of honey bee workers. They measured the activities of the enzymes -amylase, glucoamylase, trehalase, maltase, and sucrase. Their findings demonstrated that vitamin C supplementation had a beneficial effect by significantly raising glycogen and trehalose concentrations in the early stages of development and in newly emerged workers.

Seyfi *et al.,* (2019) examined the effects of vitamin C supplementation on different stages of queen rearing as well as on their maturation, mating age, and onset of oviposition. Their findings showed that colonies fed with vitamin C had an earlier onset of the egg-laying stage.

In overwintering honey bee colonies naturally infested with the parasitic mite *Varroa destructor*, Lopienska-Biernat *et al.,* (2019) investigated the effects of vitamin C supplementation on total carbohydrate content, glycogen, trehalose, glucose, and fructose concentrations, and amylase and disaccharidase activity in a developing worker brood. The results showed that vitamin C supplementation improved carbohydrate metabolism and reduced infection rates.

The effects of supplemental feeding on colony development, protein content, and antioxidant system in newly emerging workers were studied by Tawfik *et al.,* (2020). During the winter season, worker bees provided with supplementary diet showed significant improvement in colony growth metrics and antioxidant system compared to the control group. Newly hatched worker bees showed an increase in the activity of the enzymes

superoxide dismutase (SOD) and catalase (CAT), as well as glutathione (GSH) and lipid peroxidation (LPO). Barascou *et al.,* (2021) studied the effects of pollen supplementation on pesticide-induced toxicity. They found that pollen intake reduced lethality from acute pesticide doses. This reduced pesticide toxicity was associated with increased gene expression of vitellogenin. According to their results, pollen quality may influence the ability to metabolise pesticides and survive their adverse effects.

Ahmad *et al.,* (2021) studied the influence of different supplementary feeds on colony development parameters. The treatment groups received supplementary feed (45 g soybean meal + 15 g brewer's yeast + 75 g powdered sugar + 7.5 g skim milk + 7.5 g date palm pollen + 200 ml sugar syrup with vitamin C), while the control group received sugar syrup. The results showed that the supplementary diet had a great influence on the health and development parameters of honey bees, such as the amount of pollen, the brood area covered by the workers, the population density and the honey yield.

Abo *et al.,* (2022) demonstrated the effect of supplementation of vitamins on the antioxidative system in honey bees and their results showed that vitamin supplementation had the potential to improve colony development and health during winter. There was an improvement in the antioxidative system in the honey bees that received diet with vitamin mixture to those that received the other vitamins separately (A, B, C, and E) and control ones.

Xu *et al.,* (2022) reviewed the systemic toxicity caused by neonicotinoids and the effects of different agents on neonicotinoid-induced toxicity, including vitamin C. According to this study, various agents (vitamin C, NAC, CUR, GSH, resveratrol, CAPE, and TQ) protect against neonicotinoid-induced systemic toxicity by reducing ROS signalling pathways, apoptosis, and lipid peroxidation.

Several studies have been conducted to understand the factors contributing to the decline in bee populations and to examine the impact on ecosystem services. Extensive use of neonicotinoids is one of the main factors contributing to population declines. Laboratory studies have described toxic effects of neonicotinoids on learning, memory, foraging behaviour and reproduction. Numerous toxicity studies in honeybees have shown that imidacloprid impairs memory, learning, foraging ability, metabolic physiology, and the proboscis stretch reflex, which may be detrimental to pollinating foragers. Exposure to

imidacloprid can lead to biochemical changes that negatively affect colony health and performance, as well as brood development and reproduction. The main targets of imidacloprid are the nAChRs; after binding to them, it decreases nAChR expression and alters acetylcholinesterase activity, which eventually decreases impulse transmission. In addition, imidacloprid suppressed the expression of genes related to immunity, development, detoxification and antioxidation. Few studies have been conducted to investigate the effect of ascorbic acid supplementation in honeybees. Ascorbic acid is able to increase the activity of antioxidant enzymes and of enzymes involved in carbohydrate metabolism.

Neonicotinoids have been shown to have lethal and sublethal effects in laboratory experiments and field studies. Future research studies need to use field-relevant exposure time points, concentrations, and assessment durations. The effects of neonicotinoids on the European honey bee, *Apis mellifera*, have been thoroughly studied. In contrast, there is a lack of knowledge on the Asian honey bee *Apis cerana,* which provides invaluable products, contributes to the conservation of natural resources, and pollinates agricultural crops. Future research should go beyond the classical model to include other important bee species such as *A. cerana*, which is an important component of beekeeping in Asia.

The present study hypothesises that dietary supplementation with ascorbic acid could reduce toxicity induced by sublethal concentration of imidacloprid in *Apis cerana indica*. The study examines the effects of ascorbic acid supplementation on acetylcholinesterase activity, antioxidant enzyme activities, and expression of genes related to antioxidation and reproduction.

Chapter 3.
Materials and Methods

Contents

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3.1. Experimental Organism

Apis cerana indica, the Indian honey bee, was selected as the experimental animal for the present study. It is a subspecies of Asian honey bee, which is one of the most abundant and domesticated bees in India. It inhabits a wide range with a variety of habitats including tropical climates, arid semi-deserts and regions at higher latitudes and altitudes. It is one of the most important pollinators of coconut palms. In southern India, they are kept for honey collection, crop pollination and increased productivity.

Honey bees were obtained from the colonies of *A. cerana indica* kept in the domestic garden (10.89 latitude, 76.4 longitude) in Palakkad district, Kerala (Figure 3.1.). Standard procedures of maintenance were followed for all colonies to ensure the presence of sufficient number of adult workers, drones and sealed worker and drone cells. The colonies were inspected regularly and care was taken to avoid infections and diseases.

Systematic position of *Apis cerana indica*

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Hymenoptera
Family	:	Apidae
Genus	:	*Apis*
Species	:	*cerana*
Sub species	:	*indica*

Figure 3.1. Colonies of ***A. cerana indica*** maintained in the domestic garden (latitude-10.89, longitude-76.4) in Katampazhipuram, Palakkad District, Kerala.

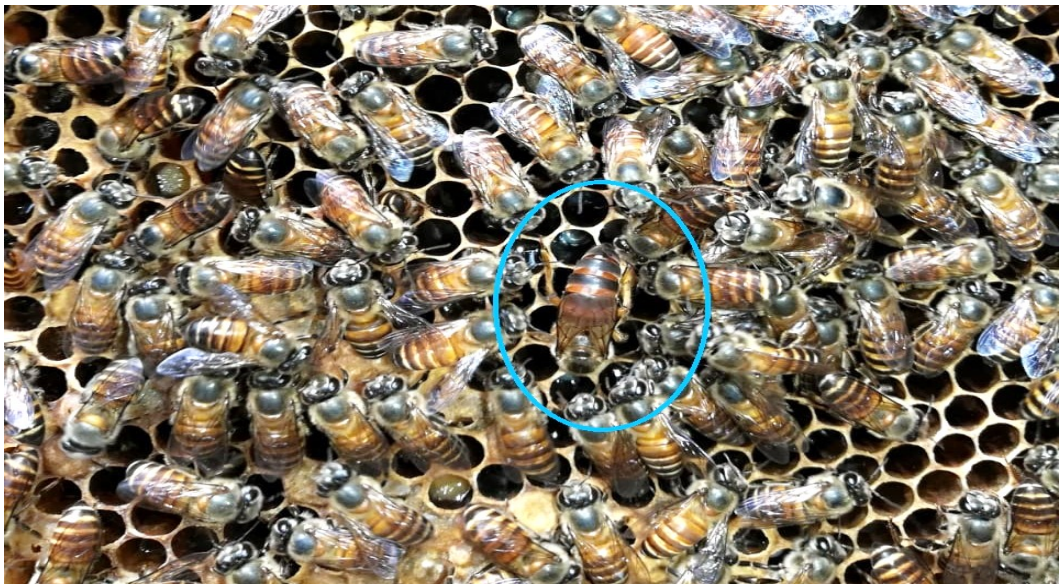


Figure 3.2. Honey bee workers and Queen (in circle) of *Apis cerana indica*.

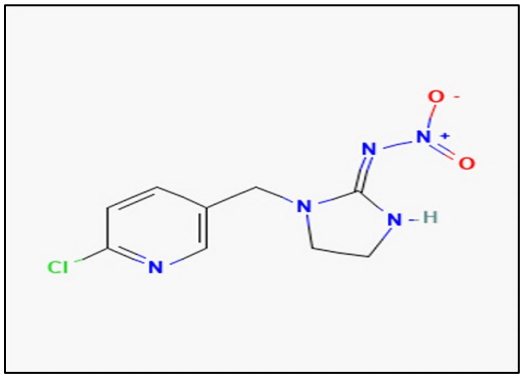
3.2. Selection of Pesticide

Imidacloprid was selected as the pesticide for the present study. Imidacloprid is a systemic insecticide that belongs to the group of neuroactive insecticides called neonicotinoids. Products containing imidacloprid are widely used for various purposes. It is used as a systemic insecticide, a spray insecticide, a termiticide, a flea control agent, and a pest control agent. Imidacloprid is a topical insecticide that can also be applied as a soil injection, tree injection, foliar application or pesticide-coated seed. It is available under the brand names Confidor, Admire, Gaucho, Antarc, Chinook, Premise, Faibel, etc. The present study was carried out with imidacloprid (analytical grade) purchased from Sigma-Aldrich (Mumbai, India).

Chemical properties of Imidacloprid

Molecular formula	: $C_9H_{10}ClN_5O_2$
Molecular Weight	: 255.66
CAS Number	: 138261-41-3
Melting point	: 141-146 C
Solubility in water	: 0.51 g/L (20 °C)
Drug category	: Agrochemicals, Cholinergic agents.
Chemical category	: Nitroguanidines.
IUPAC name	: N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl] nitramide

Figure 3.3. Chemical structure of Imidacloprid



3.3. LD_{50} determination

The acute oral toxicity test was conducted according to Medrzycki *et al.,* (2013) to determine the toxicity of imidacloprid to worker bees. To determine the LD_{50}, worker bees were randomly collected from a single colony of *A. cerana indica* in clean disposable containers. The disposable containers used for the experiments were constructed according to the minimum requirements for maintaining adult worker bees in the laboratory as described by William *et al.,* (2013) and Evan *et al.,* (2009) with slight modifications. The disposable containers have a height of 15 cm, a bottom diameter of 5 cm, and a top diameter of 9 cm. They were provided with a lid with evenly spaced ventilation holes on the sides. A larger hole in the lid was used to accommodate a 1.5-ml feeding tube for providing sugar syrup.

The acute oral toxicity test was performed according to OECD-213 (1998) guidelines. Six groups (six containers) including a control group with 20 bees in each group were kept for the study. The bees were starved for one hour before the test. The control group received 1 ml of sugar syrup and the test groups received 1 ml of sugar syrup with different doses of imidacloprid (2.5 ppm, 5 ppm, 7.5 ppm, 25 ppm, 50 ppm) and were kept at room temperature. The number of bees with behavioural changes and the percentage of mortality were monitored regularly throughout the experiment. The mortality rate after 24 hours and the amount of sugar syrup consumed per group were recorded regularly. For the calculation of ingested dose, the feeding tube containing the test solution was weighed before and after the exposure. The above experiment was repeated three times by using worker bees from different colonies in order to confirm the rate of mortality.

Mortality data were collected and analyzed to determine the LD_{50}. The lethal dose for 50% mortality was calculated based on a probit analysis for 24 hours with a confidence limit of 5%. Based on the observed percent mortality of honey bees, the imidacloprid concentration at which no mortality occurred after 24 hours of exposure was selected for further exposure experiments. From the percent mortality data, the sublethal concentration of 1µg/ml (1ppm) imidacloprid was selected for the study. Analysis of biochemical parameters in worker bees, determination of antioxidant enzymes in hemolymph and midgut,and histopathology of reproductive and non-reproductive tissues were evaluated after a one-hour exposure to 1 µg/ml imidacloprid.

3.4. Experimental protocol

3.4.1. Field colonies used in the experiments

Six healthy colonies of *A. cerana indica* were selected from the apiary in the domestic garden. The colonies were divided into control and experimental colonies (Figure 3.5). The presence of queen, worker bees, drones and larvae was ensured in all colonies and regularly checked for colony viability. The control colonies were provided with 250 ml of sugar syrup (1:1 sugar and water) and the experimental colonies were provided with 0.2% ascorbic acid added to the 250 ml of sugar syrup. The colonies were fed once a week throughout the experimental period (June-December). For the present experiment, the concentration of ascorbic acid (0.2%) was achieved by conducting preliminary experiments using different concentrations of ascorbic acid.

3.4.2. Multiple queen rearing

Multiple queens were produced from a control colony and an experimental colony by a new method described by Syama and Sreeranjitkumar, (2021). The selected colonies were divided to create queenless colonies. Regular observations were made to ensure that worker bees in queenless colonies were building better queen cells. Mature queen cells (mature queen cells in which queens are ready to hatch) were removed from the comb with utmost care on the eighth day after colony division (Figure 3.6. A). The removed queen cells were placed in queen cups attached to a free frame. All queen cups were then closed with queen cages (Figure 3.6. B). The frames with queen cages were placed in the same queenless colony (Figure 3.6. C). The queen cells were observed regularly to monitor the emergence of the queen. The newly hatched queens from the control colony and the experimental colony were transferred to the control colonies and the experimental colonies in the garden. These queens of the same age were used to conduct laboratory experiments.

3.4.3. Laboratory experiments

Queen bees and worker bees were collected from control and experimental colonies in small cellophane containers which are provided with a lid and ventilation holes on the sides (Figure. 3.7). A larger hole in the lid was used to accommodate a feeding tube for the sugar syrup. The queen bees and worker bees from the control colonies were divided into two

groups. The pictorial description of the methodology is shown in Figure 3.4. One group received only sugar syrup (control) and the other group received sugar syrup together with imidacloprid at a concentration of 1 µg/ml (IMD). The experimental colony supplemented with ascorbic acid was also divided into two groups, one fed with sugar syrup only (VIT C Control) and the other with sugar syrup together with IMD at a concentration of 1 µg/ml (VIT C + IMD) (Figure 3.8). The feeding tube containing the test solution was weighed before and after the exposure period to calculate the ingested dose.

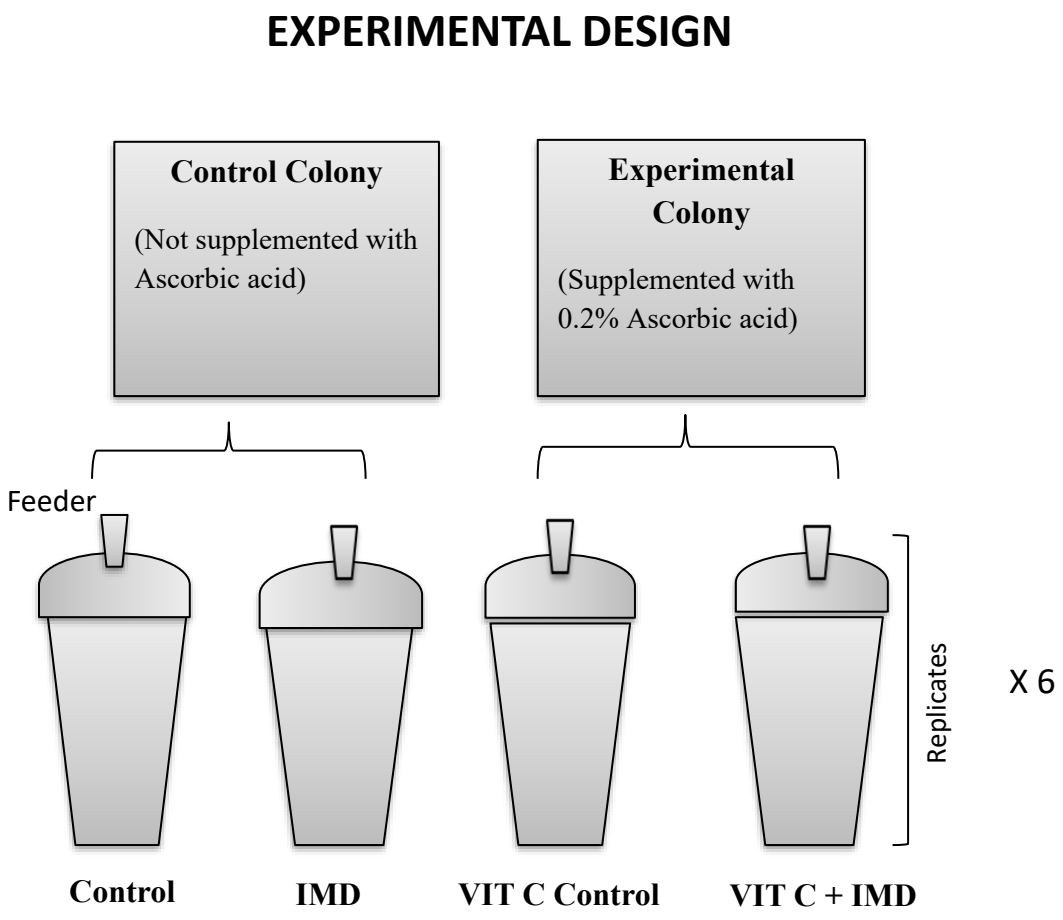


Figure 3. 4. Experimental design. Each cage contained 20 worker bees and one Queen Bee. Sugar syrup and IMD were provided through feeders. **Control:** Queen bee and worker bees from control colony, **IMD:** Queen bee and worker bees from control colony exposed to IMD at 1µg/mL, **VIT C Control:** Queen bee and worker bees from Experimental colony, **VIT C + IMD:** Queen bees and worker bees from Experimental colony exposed to IMD at 1µg/mL.

Figure 3.5. Control Colonies and Experimental colonies of ***A. cerana indica*** maintained in the domestic garden. **A: Control** colonies of ***A. cerana indica*** provided with sugar syrup. **B: Experimental colonies** of ***A. cerana indica*** supplemented with 0.2% ascorbic acid.

Figure 3.6. Multiple Queen rearing from queenless colonies. **A.** Mature queen cells, **B.** Mature queen cells capped with queen cages, **C**. Queen cages inserted in to the queenless colony, **D**. Emergence of queen.

Figure 3.7. Capturing Queen bees and worker bees for conducting laboratory experiments

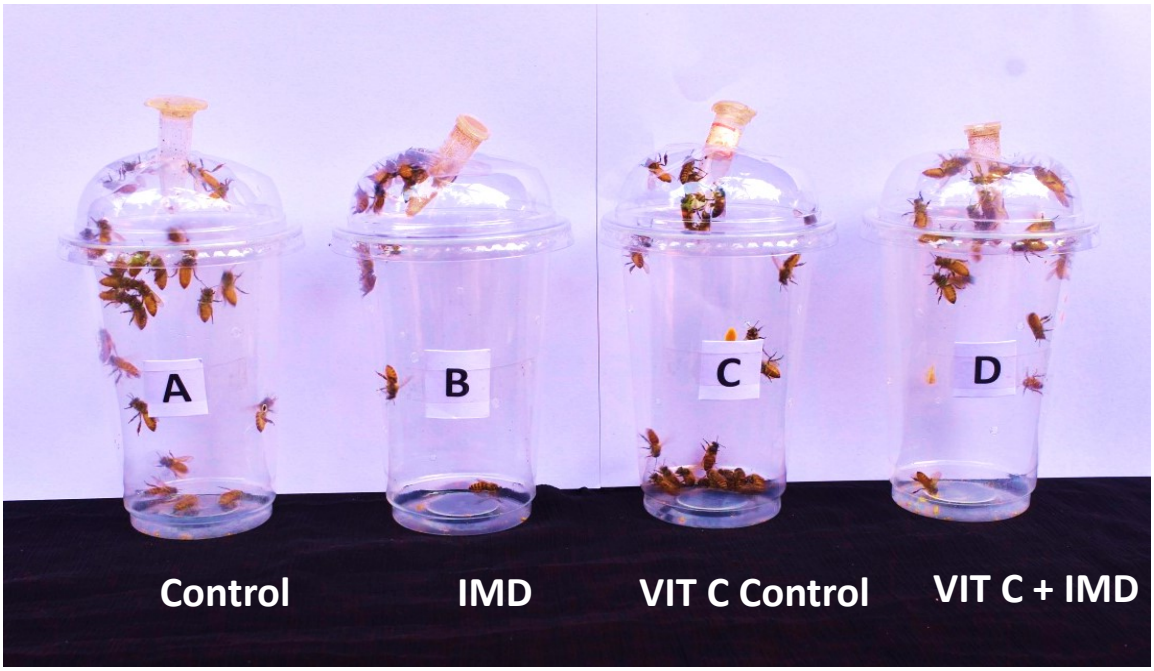


Figure 3. 8. Queen bees and worker bees collected from the control and experimental colonies for laboratory exposure experiments. **Control:** Queen and workers from control colony, **IMD:** Queen and workers from Control colony exposed to IMD at 1µg/mL, **VIT C Control:** Queen and workers from experimental colony, **VIT C + IMD:** Queen and workers from experimental colony exposed to IMD at 1µg/mL.

3.5. Sample collection

At the end of each exposure period, the body weight of queen bees was immediately measured. Samples were collected by anesthetizing the bees at 4°C for five minutes. The bees were immediately dissected and the crude extract from the corresponding tissues of the worker bees was used to measure the activities of the enzymes. For each crude extract, a specific tissue was taken, weighed, and the appropriate amount of PPT buffer was added. Insect saline (7.5 g NaCl, 2.38 g Na_2HPO_4, and 2.72 g KH_2PO_4 in 1L distilled water) was used for the preparations. The respective tissues were prepared according to the requirements of the parameters. Cold condition was maintained during the collection and the samples were stored at -20 °C.

3.5.1. Collection of hemolymph

The worker bees of each group were anesthetized by keeping them at 4°C for five minutes. Hemolymph was collected using a microcapillary tube by inserting a sterile needle dorsally between the 5^{th} and 6^{th} abdominal segments by using a sterile needle. Collected hemolymph was pooled in pre-cooled Eppendorf tubes with an equal volume of PPT buffer (100mM potassium phosphate buffer containing 0.01% phenylthiourea). Samples were centrifuged at 10,000 rpm for 20 minutes at 4º C and used for analysis of various biochemical parameters and enzyme assays.

3.5.2. Collection of tissues: worker bee

The digestive tract of the worker bee was dissected out following the protocol explained by Carreck *et al.,* (2013). The abdomen was opened by an incision across the tergite, and the roof of the abdomen was lifted off without damaging the internal organs. The alimentary canal was elevated by passing a needle under the rectum and ventriculus. The alimentary canal was carefully removed, rinsed in insect saline, and the fat bodies removed. The midgut was carefully excised and homogenized in saline, centrifuged at 10,000 rpm for 20 minutes at 4º C, and the supernatants used for enzyme assays. The heads and midgut of worker bees from each group were dissected in insect saline and then fixed in Bouin fixative for histological analysis.

The brain of the frozen worker bee was removed following the protocol described by Carreck *et al.,* (2013). The upper mask was lifted by cutting around the edge of the compound eyes. The compound eyes were removed at the level of the optic lobes. The brain was removed with a sterile scalpel and forceps, rinsed in ice-cold phosphate buffer (pH 7), and immediately homogenized with an extraction solution of 10 mM NaCl, 1% Triton 100, and 40 mM sodium phosphate buffer (pH 7.4). The homogenizate was centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatant was used for the determination of AChE and protein estimation.

To perform molecular analysis, brain tissue was immediately transferred to a 50-ml tube containing TRIzol, homogenized and were kept at room temperature for 5 minutes. The supernatant was carefully transferred to fresh, RNase-free tubes and centrifuged at 10,000rpm for 15 minutes at 4°C for mRNA extraction.

3.5.3. Collection of tissues: Queen bee

Queen from each group were immediately dissected (Carreck *et al.,* 2013) in sterile cold conditions. Mounted the queen by keeping the dorsal surface uppermost. Opened the abdomen without damaging the internal organs. Gripped the ventriculus using forceps and removed the alimentary canal without damaging the ovaries. Ovaries are lifted up using a sterilized needle. Midgut tissues and ovaries were fixed in Bouin's fixative for histological analysis.

For molecular analysis, ovaries were immediately transferred to a 50ml tube containing the TRIzol, homogenized and kept the samples for 5 minutes at room temperature. The supernatant was carefully transferred to fresh, RNase free tubes and were centrifuged at 10,000rpm for 15 minutes at 4^0C for mRNA extraction.

3.6. Chemicals

Imidacloprid, Bovine serum albumin, trichloroacetic acid, Trizol, cDNA synthesis mix, was procured from Sigma-Aldrich (Mumbai, India). NADP, NADPH, Reduced Glutathione, Oxidised Glutathione, Mercaptoethanol, Hydrogen peroxide, RT-PCR master mix and Primers was purchased from Himedia Laboratories, Mumbai, India. All other chemicals were of analytical grade and procured from local commercial sources.

3.7. Biochemical analysis of the hemolymph

3.7.1. Estimation of total soluble protein

Total soluble protein in hemolymph was analyzed using Lowry's method (Lowry *et al*., 1951).

Reagents

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 1% Na-K Tartrate in H_2O

C. 0.5% $CuSO_4.5\ H_2O$ in H_2O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml of C

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part distilled water

F. BSA Standard - 10 mg BSA dissolved in 100 ml of distilled water

Procedure

BSA working standard of known concentration was used for the preparation of a standard curve. Experimental samples were taken in test tubes and were made up to 1 ml using distilled water. A test tube containing 1 mL distilled water served as a blank. 4.5 ml of Reagent I (48 ml of 2% Na_2CO_3 in 0.1 N NaOH , 1 ml of 1% Na-K Tartrate in H_2O , 1 ml of 0.5% $CuSO_4.5\ H_2O$ in H_2O) was added and incubated for 10 minutes. After incubation, 0.5ml of Reagent II (1 part Folin-Phenol [2 N]: 1 part water) was added and incubated for 20 minutes. A standard graph was plotted after measuring the absorbance at 650nm. The standard graph was used to estimate the amount of protein present in the samples using the formula.

$$\text{Concentration of unknown} = \frac{\text{OD of unknown}}{\text{OD of standard}} \times \text{Concentration of Standard}$$

3.7.2. Estimation of total Carbohydrate

Total carbohydrate content in the hemolymph of worker bees was determined by using the Anthrone method (Hedge and Hofreiter, 1962).

Reagents

1. 2.5 N-HCl
2. Working standard: 10mL of stock diluted to 100mL with distilled water

3. Anthrone reagent : dissolved 100mg anthrone in 50ml of 95% H_2SO_4

Procedure

Hydrolyzed the samples with 5mL of 2.5 N-HCl by keeping it in a boiling water bath for 3 hours and cooled to room temperature. The samples were centrifuged and collected from the supernatant for analysis. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1mL of the working standard. Then added 4mL of freshly prepared anthrone reagent in all tubes including sample tubes and heated for eight minutes in a boiling water bath. Cooled rapidly and optical density was measured at 630nm.

3.7.3. Activity of Glucose-6-phosphate dehydrogenase (G6PD)

The colorimetric assay explained by Balinsky and Bernstein, (1963) was adopted to assay the activity of glucose 6 –Phosphate dehydrogenase.

Reagents

1. 0.1 M Tris- Hcl buffer (pH 8.2)
2. 0.2mM NADP
3. 0.1M Magnesium chloride
4. 6mM Glucose 6 Phosphate

Procedure

0.5 ml of enzyme extract was added to a sample cuvette containing the reaction mixture containing 0.5 ml Tris- Hcl buffer, 0.5 ml of NADP, 0.5 ml of magnesium chloride, and 1ml of distilled water. Incubated for 1 minute and the increase in the absorbance were measured at 340 nm. Reaction mixture without enzyme was used as a reference blank. The activity of the enzyme was calculated using the formula.

$$\text{Specific activity (µmoles/min/mg protein)} = \frac{\Delta A \times TV \times 1000}{K \times \text{Volume of sample} \times \text{mg protein}}$$

($\Delta A = (F_A - I_A) / (F_T - I_T)$, Total volume= 3 ml, Volume of sample= 0.5 ml).

3.7.4. Activity of Acetylcholinesterase (AChE)

Reagents

1. Acetylthiocholine iodide, 0.10 M in sodium phosphate buffer (0.1 M, pH 8.0)

2. DTNB, 0.01 M in sodium phosphate buffer (0.1 M, pH 8.0 containing 1.5% sodium carbonate)

3. Sodium phosphate buffer (0.1 M, pH 8.0)

Procedure

AChE activity was determined using the colorimetric method described by Kranthi, (2005). 2.86 ml sodium phosphate buffer (0.1 M, pH 8.0) was taken in a 4 ml cuvette (sample cuvette) and 100 µl of the enzyme was added and was incubated for 5 minutes. 10 µl of the DTNB solution, 30 µl of Acetylthiocholine Iodide were added and incubated at room temperature for 3 minutes. In another cuvette, 2.96 ml of sodium phosphate buffer, 10 µl DTNB and 30 µl Acetylthiocholine Iodide solutions were taken and kept as blank and recorded the increase in absorbance in the sample at 412 nm for 3 minutes. The activity of the enzyme was calculated using the formula.

$$\text{AChE activity (µmoles/min/mg protein)} = \frac{\Delta A \times 1000 \times 3.0}{1.36 \times 10^4 \times 0.10 \times \text{mg protein}}$$

Where,

ΔE = change in absorbance per minute.

3.0 = total volume of reaction mixture (ml).

0.1 = volume of enzyme (ml).

1.36×10^4 = molar extinction coefficient

3.8. Antioxidant enzyme assays

3.8.1. Catalase (CAT)

The UV-spectrophotometric method described by Luck, (1963) was adopted to measure the activity of catalase.

Reagents

1. Sodium phosphate buffer (0.01 M, pH7.0)
2. Disodium hydrogen phosphate- 0.2 g and Sodium dihydrogen phosphate- 0.05 g dissolved in 100 ml distilled water.

Procedure

1.5 ml of H_2O_2 and 1.455µl of 0.01M phosphate buffer (pH 7.0) of reaction mixture was taken in a sample cuvette and 45 µl of the enzyme extract was added and the OD change was measured at 240 nm for 3 minutes. 2ml of reaction mixture was taken in a cuvette as reference blank.

$$\text{Specific activity (µmoles/min/mg protein)} = \frac{\Delta A \times TV \times 1000}{K \times V \text{ of sample} \times \text{mg protein}}$$

($\Delta A = (F_A - I_A) / (F_T - I_T)$, Total volume= 3 ml, Volume of sample= 0.045 ml, K = 6.93 x $10^{-3} mM^{-1} cm^{-1}$).

3.8.2. Peroxidase (POD)

The peroxidase (POD) activity was determined spectrophotometrically by the method proposed by Reddy *et al*., (1995) using catechol as substrate. The increase in the absorption caused by oxidation of catechol by H_2O_2, was measured at 420 nm.

Reagent preparation

1. Sodium phosphate buffer (0.1 M, pH 6.5)- Disodium hydrogen phosphate- 1.65 g and Sodium dihydrogen phosphate- 0.53 g dissolved in 100 ml distilled water.
2. 0.005 M catechol- 0.06 gm catechol in 100 ml distilled water
3. Hydrogen peroxide

Procedure

0.05ml of the enzyme extract was added to the reaction mixture containing, 2.5 ml of 100 mM sodium phosphate buffer (pH 7.0), 0.25 ml of 5 mM catechol and 0.20 ml of 5 mM H_2O_2. 2ml of reaction mixture was taken in a cuvette as reference blank and the change in absorbance was recorded for 3 minutes at 420nm.

The activity of POD was calculated using the formula,

$$\text{Specific activity (µmoles/min/mg protein)} = \frac{\Delta A \text{ x } T.V \text{ x } 1000}{K \text{ x } V \text{ of sample x mg protein}}$$

($\Delta A = (F_A - I_A) / (F_T - I_T)$, Total volume= 3ml, K for catechol oxidase=0.272, Volume of sample= 0.2 ml)

3.8.3. Glutathione-S-Transferase (GST)

Reagent preparation

1. Reduced glutathione 50 mM in phosphate buffer (100 mM, pH 6.5).
2. CDNB 50 mM in ethanol
3. Sodium Phosphate buffer (100 mM, pH 6.5), containing 1 mM EDTA.

Procedure

Glutathione S-transferase was assessed spectrophotometrically (Habig *et al*., 1974) by adding 50 µl of 50 mM CDNB, 150 µl 50 mM reduced glutathione to 2.77 ml Phosphate buffer (100 mM, pH 6.5). 30 µl of enzyme extract was added to this reaction mixture and incubated for 3 minutes. 3 ml of reaction mixture without the enzyme was kept as blank and the absorbance was recorded for 3 minutes at 340 nm. Enzyme activity was calculated using the formula

$$\text{Specific activity (µmoles/min/mg protein)} = \frac{\Delta A \times T.V \times 1000}{K \times \dot{V} \text{ of sample} \times \text{mg protein}}$$

($\Delta A = (F_A - I_A) / (F_T - I_T)$, Total volume= 3ml, K = 9.6 mM^{-1} cm^{-1}, Volume of sample= 0.03 ml)

3.8.4. Glutathione peroxidase (GPx)

The activity of glutathione peroxidase was determined spectrophotometrically by using the method described by Rotruck *et al.,* (1973).

Reagents

1. Phosphate buffer (pH7.0, 0.4M)
2. Sodium Azide (4mM)
3. Reduced glutathione (4mM)
4. Hydrogen peroxide
5. 10% TCA
6. Disodium Hydrogen Phosphate (0.3 M)
7. DTNB – 0.04%
8. Standard reduced glutathione- 0.002%

Procedure

0.5ml Phosphate buffer, 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml H_2O_2 and 0.1 ml homogenate and 2 ml distilled water were taken in a test tube. The tubes were incubated at room temperature for 3 minutes and 0.5ml TCA was added. The suspension was

centrifuged at 3000 rpm, supernatant was decanted and 4.0 ml of Na_2HPO_4 solution and 1 ml of DTNB reagent were added. The absorbance was read at 412 nm against a reference blank containing phosphate solution and DTNB. The enzyme activity was calculated by the following formula

$$\text{Specific activity (µmoles/ mg protein)} = \frac{\text{OD of Test x Con.of Standard x TV x 1000}}{\text{OD of Standard x Volume of enzyme x mg protein}}$$

3.8.5. Glutathione Reductase (GSR)

The activity of glutathione reductase was measured according to the method of David and Richard, (1983).

Reagents

1. Sodium phosphate buffer (0.2M, pH 7.2)
2. Oxidised Glutathione- 50mM
3. NADPH- 4mM
4. Sodium azide- 50mM

Procedure

0.1 ml sample, 1 ml phosphate buffer, 0.1 ml of EDTA, 0.1 ml sodium Azide and 0.1 ml oxidized glutathione and were taken in a test tube and the volume was made up to 3 ml with distilled water. The mixture was kept at room temperature for 3 minute and 0.1 ml NADPH was added. The absorbance read at 340 nm for 5 min against a blank containing reagent mixture without enzyme extract. Activity was calculated using the formula,

$$\text{Specific activity (µmoles/min/mg protein)} = \frac{\Delta A \text{ x TV x } 1000}{6.22 \text{ x sample volume x mg protein}}$$

3.8.6. Superoxide dismutase (SOD)

SOD activity was determined according to the spectrophotometric method of Paoletti *et al.,* (1986).

Reagents

1. Triethanolamine-diethanolamine (100mm each)-HCl Buffer: pH 7.4
2. NAD(P)H-7.5mM
3. EDTA:MnCl2 (100mM/50mM)
4. Mercaptoethanol(10mM)

Procedure

1.6ml buffer, 80 µl NAD(P)H, 50 µl EDTA:MnCl2 was taken in a cuvette and 200 µl sample was added and incubated for 5 minutes. 200 µl mercaptoethanol was added and read the absorbance at 340 nm for 3 minutes against a reagent blank.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \text{ X } 100, \qquad \text{SOD Activity (U/ml)} = \frac{\% \text{ of inhibition}}{50\%}$$

3.9. Molecular analysis

3.9.1. Total mRNA extraction

Tissue was homogenized in Trizol and centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was transferred to fresh, RNase free tubes and added 1 µl glycogen and 250µl chloroform to each Trizol supernatant and shaken for 30 seconds. After incubation at room temperature for 10 minutes, samples were centrifuged at 10,000g for 20 minutes at 4°C. Transferred the aqueous layer to RNase free tubes and added an equal volume of isopropanol to each tube. Mixed well and incubated overnight at -20°C. Centrifuged at 10,000g for 20 minutes at 4°C. Removed the isopropanol from the pellet and 1ml of cold ethanol (75%) was added. Centrifuged at 7500g for 4 minutes at 4°C. Removed the ethanol without disturbing the pellet and kept the pellet at room temperature for 5 minutes. Dissolved the RNA pellet in 100µl RNase free water (Chomczynski and Mackey, 1995).

3.9.2. cDNA Synthesis

1-5µg mRNA and 1µl prime oligo (dT) primer was added to sterile nuclease free tube on ice and added DEPC treated water to make total volume of 12.4µl. Mixed gently and incubated at 70°C for 5 minutes. cDNA synthesis mix containing 4 µl of 5x first-strand buffer, 1 µl of dNTPs (10 mM each) and 1 µl M-MLV was added, mixed gently and incubated for 60 minutes at 42^0C. Terminated the reaction by heating at 70°C for 5 min.

3.9.3. Quantitative real-time PCR assay

Quantitative real-time PCR assay was performed using the SYBR Green Fluorescent dye. The reaction mixture containing 20ng of cDNA template,10µl of RT- PCR master mix, 1µl of forward primer and 1µl of reverse primer was made up to a final volume of 20µl with nuclease free water. Negative control was kept with all reagents except sample cDNA. The

assay was performed in a 96-well PCR plate and was subjected to initial denaturation at 95°C for 5 minutes and 40 cycles of 95°C for 30s for denaturing followed by 60°C for 60s for annealing and elongation.

The primer sequences used for nicotinic Acetylcholine receptor genes in the present study were taken from literature (Christen *et al.,* 2016) and the primer sequences used for ovarian genes were designed using NCBI primer BLAST tool. Housekeeping gene Actin was used as a reference gene for the expression analysis. The details of primer sequences used for the present study are given in the **Table 3.1**

Table 3.1. Primer sequences used for quantitative PCR analysis.

Primer name	Sequence 5'> 3'	Accession number	Source	Location
βActin	Forward: TGCCAACACTGTCCTTTCTG	AB023025		Housekeeping
	Reverse: AGAATTGACCCACCAATCCA			
nAhRα1 subunit	Forward: GAAATACGTGGCGATGGTGC	NM_001098220 XM_001121970	Christen *et al.,* 2016	Head
	Reverse: GTGGTATCGTACGGCTCGG	NM_001098220 XM_001121970		
nAhRα2 subunit	Forward: CCGAACTCTACGTACCGAGC	NM_001011625 XM_392547	Christen *et al.,* 2016	Head
	Reverse: TCGAACGTCTATCTCGCACG	NM_001011625 XM_392547		
vitellogenin	Forward: AAGGTAACAACGCCATCAGG			Ovary
	Reverse: TTTGATCGCAGTTGTCGAAG			
CYP336A1	Forward: TTCGAGGCTCTCAAGGACAT			Ovary
	Reverse: TCCGGATCGAATYATCTCTGG			

3.10. Histological studies

Samples were dehydrated by passing through an isopropyl alcohol series 70%, 80%, 90%, and 100% for 2hrs each. The samples were then soaked in xylene for an hour and embedded in wax. The tissues were sectioned (4 microns) using a rotary microtome. Following the preparation of a paraffin section, the slides with sections were warmed for 10 minutes and all the wax in the slides was removed with xylene. After thorough dewaxing, the slides were passed through several grades of alcohol (90%, 80%, 70%, 60%) to remove the xylene, and then thoroughly rinsed in water. After staining with Hematoxylin for 5 minutes, slides were passed through acid-alcohol (75% alcohol and 25% HCl) and were washed for 10 minutes in running water. After passing the slides through eosin for 3 minutes, the slides were passed through several changes of alcohol, rinsed in several baths of xylene and were mounted with D.P.X. mountant. Two slides with serial stained tissue sections per group were observed under the LEICA DM 750 light microscope and compared and processed using Image J software.

3.11. Statistical analysis

Statistical analysis was carried out using the statistical software 'R' Version 4.1.1. Probit analysis as statistical method was employed for LD_{50} calculation. The significant differences among treatments were assessed by One Way ANOVA followed by pairwise analysis using DMRT (Duncan Multiple Range Test) as post-hoc test. Results of the biochemical analysis were given as mean ± Standard Deviation for six samples per group (n=6). Differences between treatments were considered to be significant at $p \leq 0.05$ against the control group and are marked with different letters in the figures. Results of the gene expression analysis were given as mean ± Standard Deviation for four samples per group (n=4). Differences between treatments were considered to be significant at $p \leq 0.05$ against the control group and are denoted with one asterisk at $0.05 > p > 0.01$ and two asterisks at $0.01 > p > 0.001$.

Chapter 4.
Results

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Chapter 4. Results

4.1. Acute toxicity of Imidacloprid

Experiments to evaluate the oral acute toxicity of IMD to adult worker bees were conducted according to OECD guidelines by administering different doses of IMD in a geometric series in sugar solution for 24 hours. We found that the lethal dose concentration that caused the death of 50% of honey bees in a group was 36.03±0.87 µg/ml (Figure 4.1). The sublethal concentration of imidacloprid, which was lower than the LD50 value, was administered to honey bees and the percentage of honey bee mortality was monitored throughout the experiment. Exposure of imidacloprid at different concentrations of 36, 18, 9, 5, 2.5 and 1 ppm showed percentage mortality of 45, 35, 30, 10, 5.2 and 0, respectively, after 24 hours (Table 4.1.). It was observed that the concentration of 1 ppm (1 µg/ml) imidacloprid showed no mortality after 24 hours. Therefore, the sub-lethal concentration of 1µg/ml (1ppm) imidacloprid was selected for the study.

Figure 4.1. Median Lethal Dose (LD_{50}-24hr) of Imidacloprid in *Apis cerana indica*

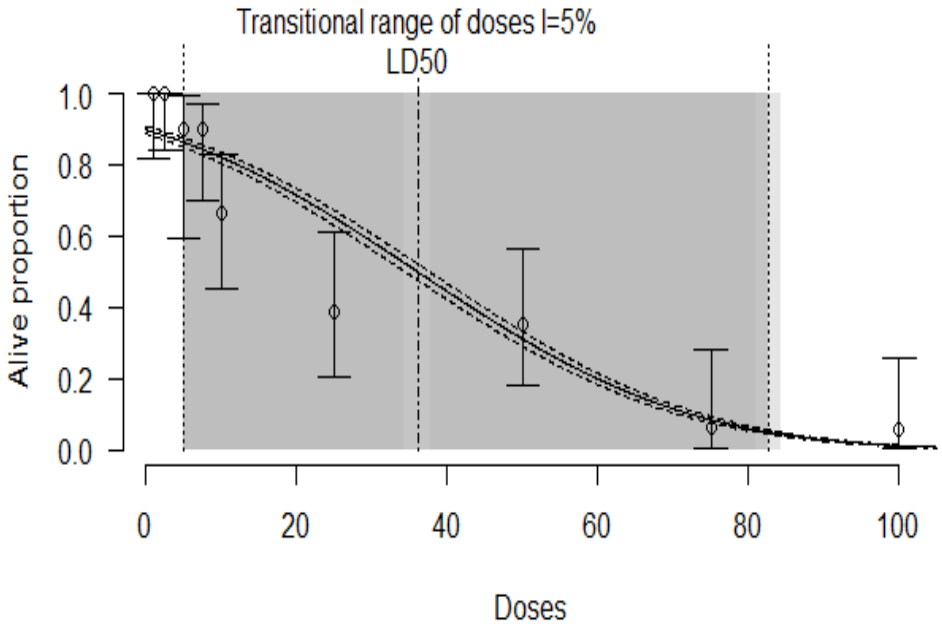


Table 4.1. Percentage mortality of honey bees exposed to different concentrations of imidacloprid (IMD) for 24 hrs.

Sl. No.	Dose (ppm)	Total No of bees	Response (mortality at 24hrs)	% of mortality
Control	**0**	**20**	**0**	**0**
1	**1**	**20**	**0**	**0**
2	**2.5**	**20**	**1**	**5.2**
3	**5**	**20**	**2**	**10**
4	**9**	**20**	**6**	**30**
5	**18**	**20**	**7**	**35**
6	**36**	**20**	**9**	**45**

4.2. Biochemical parameters

4.2.1. Total soluble protein

Total soluble protein concentration in the hemolymph of worker bees was determined by the Lowry method (Figure 4.2). The protein concentration in the hemolymph of IMD-exposed worker bees (7.95±3.1) was significantly higher ($p \leq 0.05$) than that of the control group (4.73±1.5). Compared to the control group, the group supplemented with VIT C showed a significant increase (5.40±1.7) in protein concentration. The VIT C + IMD (5.68±2.3) exposed group showed no significant difference from the VIT C supplemented group and the control groups even though there was a significant decrease in the concentration of total soluble protein in the VIT C+ IMD group compared with the IMD exposed group (Table 4.2).

Figure 4.2. Total soluble protein in the hemolymph of worker bees.

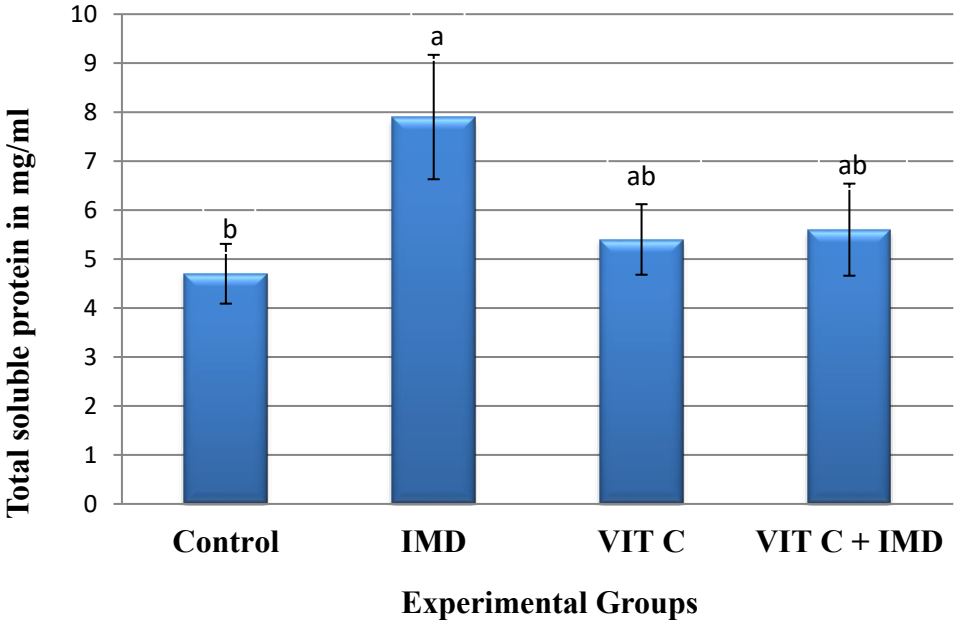


Table 4.2. Total soluble protein in the hemolymph of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Total soluble protein (mg/ml)	4.73 ± 1.5^{b}	7.95 ± 3.1^{a}	5.40 ± 1.7^{ab}	5.68 ± 2.3^{ab}

4.2.2. Total Carbohydrate content

The total carbohydrate concentration of the hemolymph of worker bees was measured (Figure 4.3). Vitamin C supplementation resulted in a significant change ($p \leq 0.05$) in total carbohydrate content (4.33±0.8). Compared to the control group (3.28±0.1), the VIT C supplemented group had a significant increase ($p \leq 0.05$) in total carbohydrate content. No significant change (2.72±0.4) in hemolymph carbohydrate content was observed with IMD exposure. The VIT C + IMD-exposed group showed a significant increase in the total carbohydrate content in the hemolymph (5.01±0.2) compared with the control, IMD, and VIT C-exposed groups (Table 4.3).

Figure 4. 3. Total Carbohydrate in the hemolymph of worker bees.

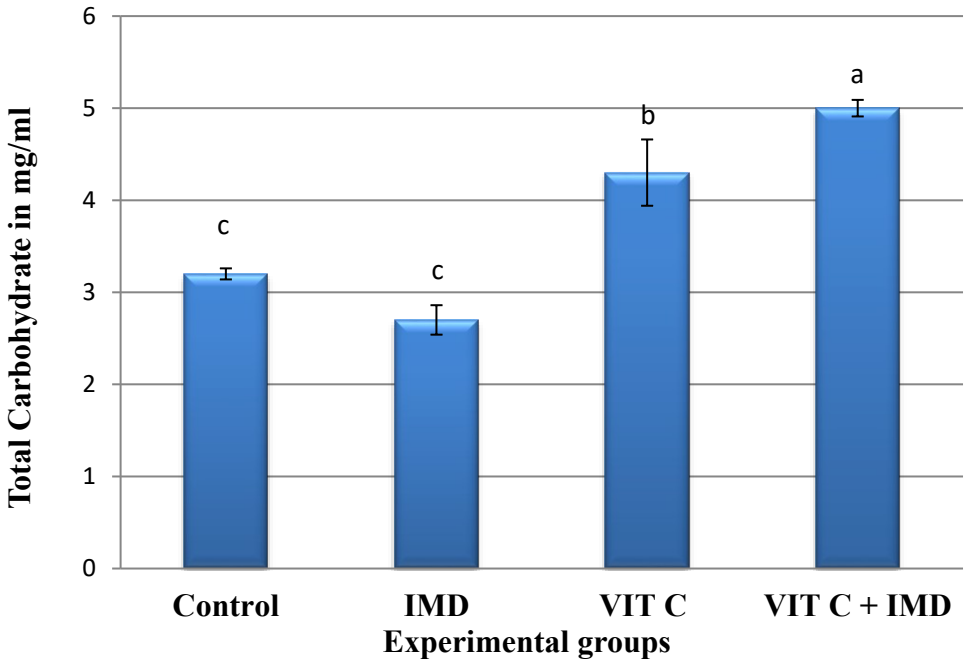


Table 4.3. Total carbohydrate in the hemolymph of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Total Carbohydrate (mg/ml)	3.28±0.1^{c}	2.72±0.4^{c}	4.33±0.8^{b}	5.01±0.2^{a}

4.2.3. Activity of Glucose 6- Phosphate Dehydrogenase

G6PD activity was measured in the hemolymph of worker bees (Figure 4.4). IMD treated bees showed lower enzyme activity (0.09±0.03) than the control group (1.19±0.2). Vitamin C supplementation (3.41±1.0) caused a significant change in G6PD activity. Compared with the control group, a significant increase in G6PD activity was observed in the VIT C-supplemented groups. The VIT C+ IMD-exposed group showed a significant increase in G6PD activity (4.7±1.9) compared with the control, IMD-exposed, and VIT C groups (Table 4.4).

Figure 4. 4. Specific activity of G6PD in worker bees of *A. cerana indica*.

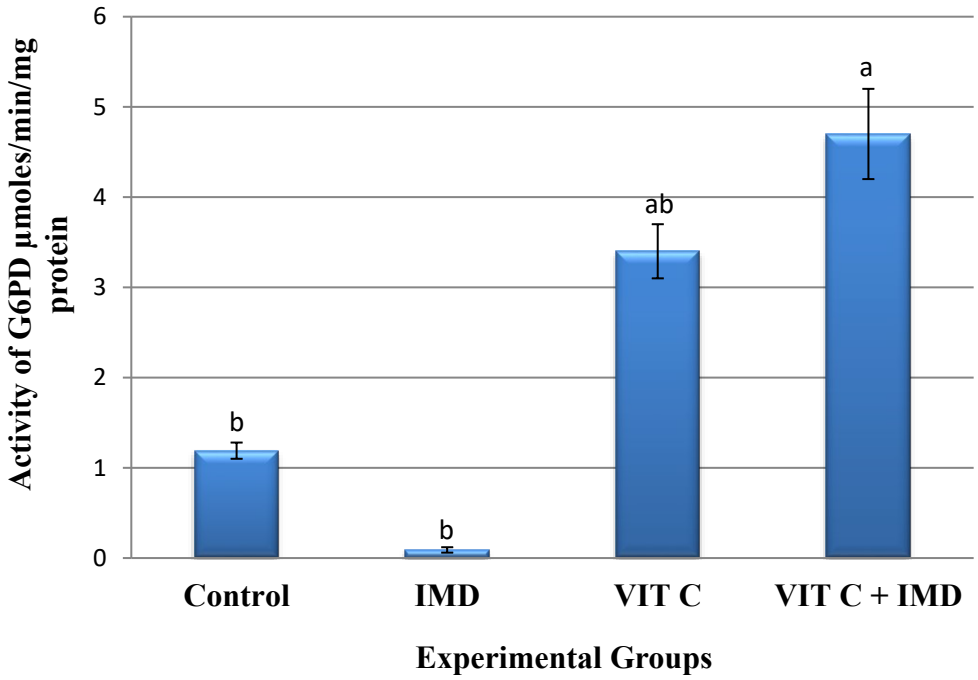


Table 4.4. The activity of G6PD in the hemolymph of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Activity of G6PD (µmoles/min/mg/protein)	1.19±0.2^{b}	0.09±0.03^{b}	3.41± 1.0^{ab}	4.7±1.9^{a}

4.2.4. Activity of Acetylcholinesterase (AChE)

AChE activity was measured in the brain of worker bees. A significant decrease in AChE activity was observed in the brain when exposed to imidacloprid alone (IMD) (0.82±0.7) compared to control (3.08±1.8) (Figure 4.5). On the other hand, the group which was subjected to the administration of VIT C as a single agent resulted in a significant increase in AChE levels (2.79±1.3) compared to the group administered IMD only. The group receiving VIT C + IMD showed a significant increase in AChE activity (1.96±1.1) compared with the group receiving IMD alone (Table 4.5).

Figure 4.5. Specific activity of AChE in worker bees of *A. cerana indica*.

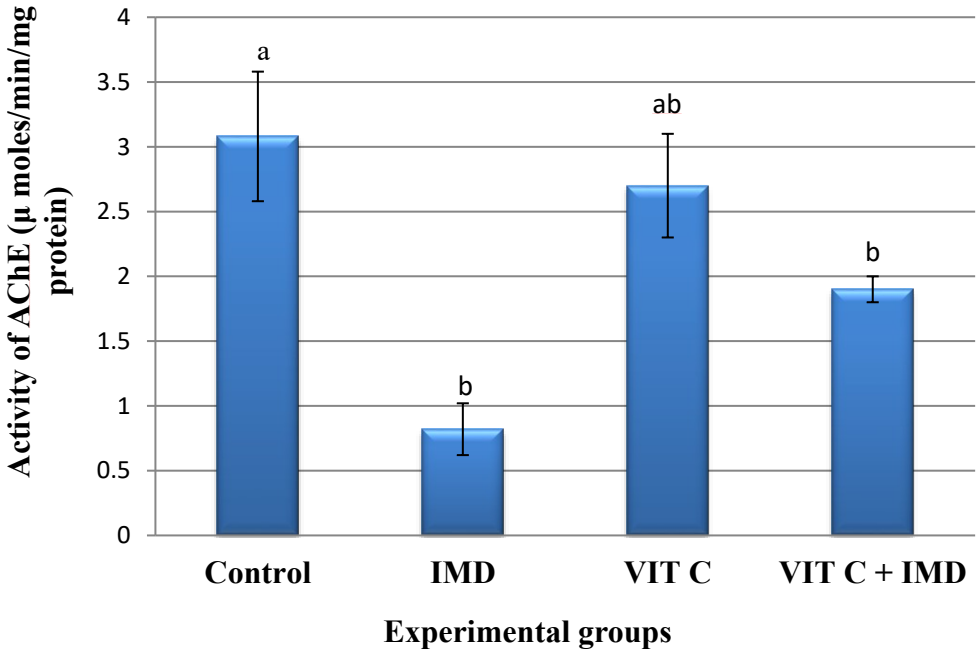


Table 4.5. Specific activity of AChE in worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Activity of AChE (µmoles/min/mg/protein)	3.08±1.8[a]	0.82±0.7[b]	2.79± 1.3[ab]	1.96±1.1[b]

4.3. Antioxidative system

4.3.1. Activity of Catalase (CAT)

Exposure of honeybees to IMD significantly inhibited catalase activity in hemolymph and midgut, as in this treatment CAT activity was 4.7±2.5 and 57.9±32.2, respectively (Figure 4.6). The activity of CAT decreased significantly compared with the control group (12.06±8.2 and 101.1±28.9 for hemolymph and midgut, respectively). When the diet was supplemented with VIT C, the CAT activity (34.9±14.5 for hemolymph and 146.2±98.1 for midgut) showed a significant increase, while a significant reduction was observed in the VIT C + IMD exposed group compared to the VIT C group, but it is in par with the control in both hemolymph and midgut (Table 4.6).

Figure 4.6. Specific activity of CAT in the hemolymph and mid gut tissues of worker bees.

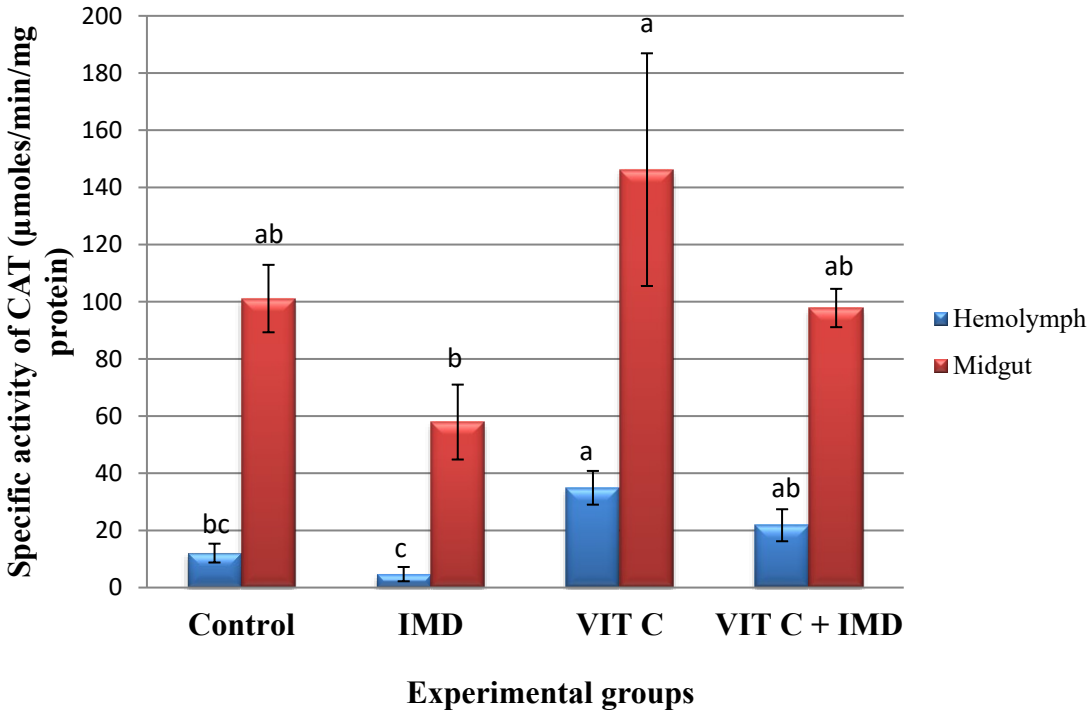


Table 4. 6. The activity of CAT in the hemolymph and mid gut of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Hemolymph	12.06±8.2^{bc}	4.7±2.5^{c}	34.9± 14.5^{a}	21.8±13.8^{ab}
Midgut	101.1±28.9^{ab}	57.9±32.2^{b}	146.2±98.1^{a}	97.8±16.6^{ab}

4.3.2. Activity of Superoxide dismutase (SOD)

Superoxide dismutase activity (SOD) was determined in both hemolymph and midgut (Figure 4.7). No significant change ($p \geq 0.05$) in the activity of SOD was observed in the hemolymph of worker bees when exposed to IMD (2.0±0.005) compared to control (2.0±0). In midgut tissues, IMD treatment resulted in a significant ($p \leq 0.05$) decrease in the activity (2.01±0.008) of SOD. A significant increase in the activity of SOD in the hemolymph and midgut of worker bees was observed in the group supplemented with VIT C (2.06±0.008 for the hemolymph and 2.04±0.02 for the midgut, respectively) compared to the control (2.0±0 for the hemolymph and 2.03±0.01 for the midgut). In the VIT C + IMD-treated group, SOD activity was significantly increased in the hemolymph (2.03±0.01) compared with the control, but no significant difference was observed in the midgut compared with the control.

Figure 4.7. Specific activity of SOD in the hemolymph and mid gut tissues of worker bees.

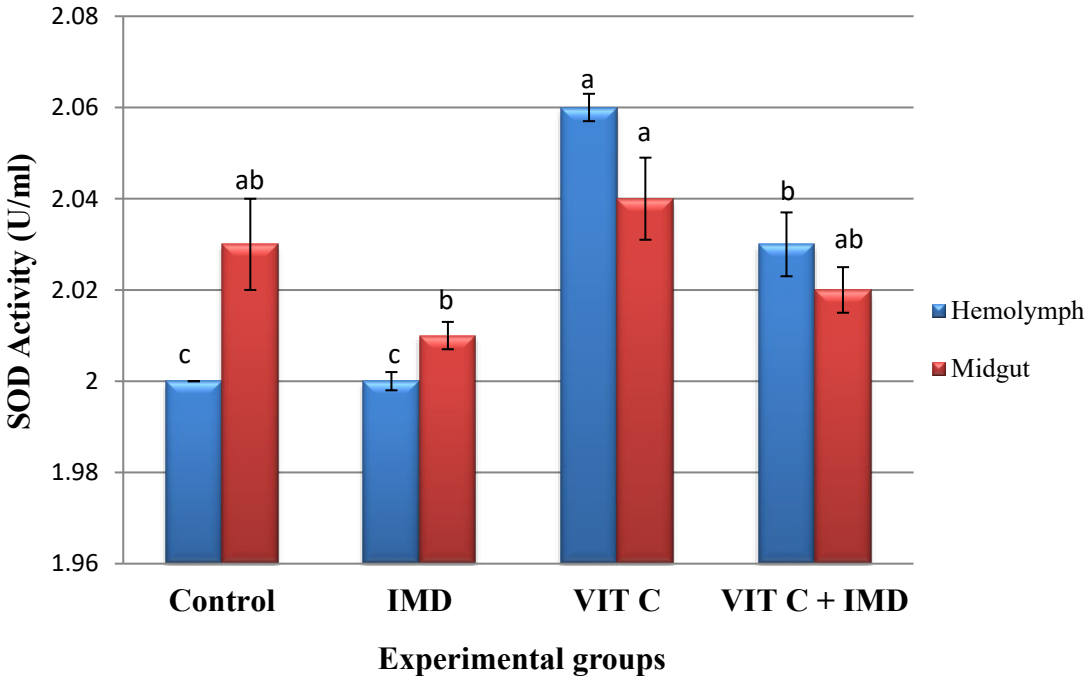


Table 4. 7. The activity of SOD in the hemolymph and mid gut of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Hemolymph	2.00 ± 0^{c}	2.00 ± 0.005^{c}	2.06 ± 0.008^{a}	2.03 ± 0.01^{b}
Midgut	2.03 ± 0.03^{ab}	2.01 ± 0.008^{b}	2.04 ± 0.02^{a}	2.02 ± 0.01^{ab}

4.3.3. Activity of Peroxidase (POD)

Peroxidase activity (POD) was examined in both hemolymph and midgut tissues of worker bees (Figure 4.8). No significant difference ($p \geq 0.05$) was found in the activity of POD in the hemolymph of worker bees from treated groups and the control. An increase in activity was observed in IMD exposed bees (2846±1223), but no significant difference ($p \geq 0.05$) was found in the other treatments. IMD treatment resulted in a significant increase ($p \leq 0.05$) in enzymatic activity in the midgut of worker bees (11660±4227) compared to control (2837±44.1). A significant decrease in the activity POD was observed in the midgut of worker bees in VIT C (3055±520) and VIT C + IMD (5670±1261) treatment compared to IMD (11660±4227) treatment (Table 4.8).

Figure 4.8. Specific activity of POD in the hemolymph and mid gut tissues of worker bees.

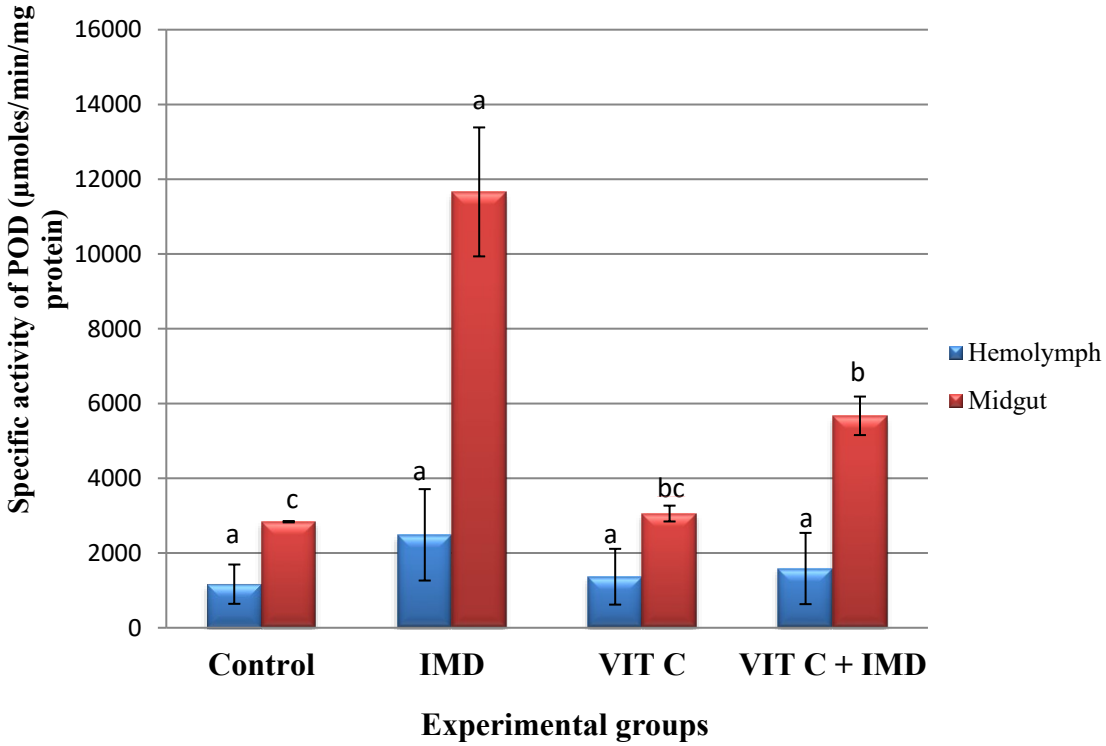


Table 4.8. The activity of POD in the hemolymph and mid gut of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Hemolymph	1166±526^{a}	2486±1223^{a}	1365±746^{a}	1585±953^{a}
Midgut	2837±44.1^{c}	11660±4227^{a}	3055±520^{bc}	5670±1261^{b}

4.3.4. Activity of Glutathione S-transferases (GST)

Worker bees in the IMD-exposed group had significantly ($p \leq 0.05$) lower GST activity in both hemolymph (2.24±1.4) and midgut (0.7±0.3) compared to their concerned control (6.45±2.6 and 1.01±0.3, respectively) (Figure 4.9). The worker bees from VIT C treatment (11.4±5.3 and 2.0±0.3) and VIT C + IMD treatment (7.44±2.3 and 2.6±0.7) had significantly ($p \leq 0.05$) higher GST activity in both hemolymph and midgut compared to control (Table 4.9).

Figure 4.9. Specific activity of GST in the hemolymph and mid gut tissues of worker bees.

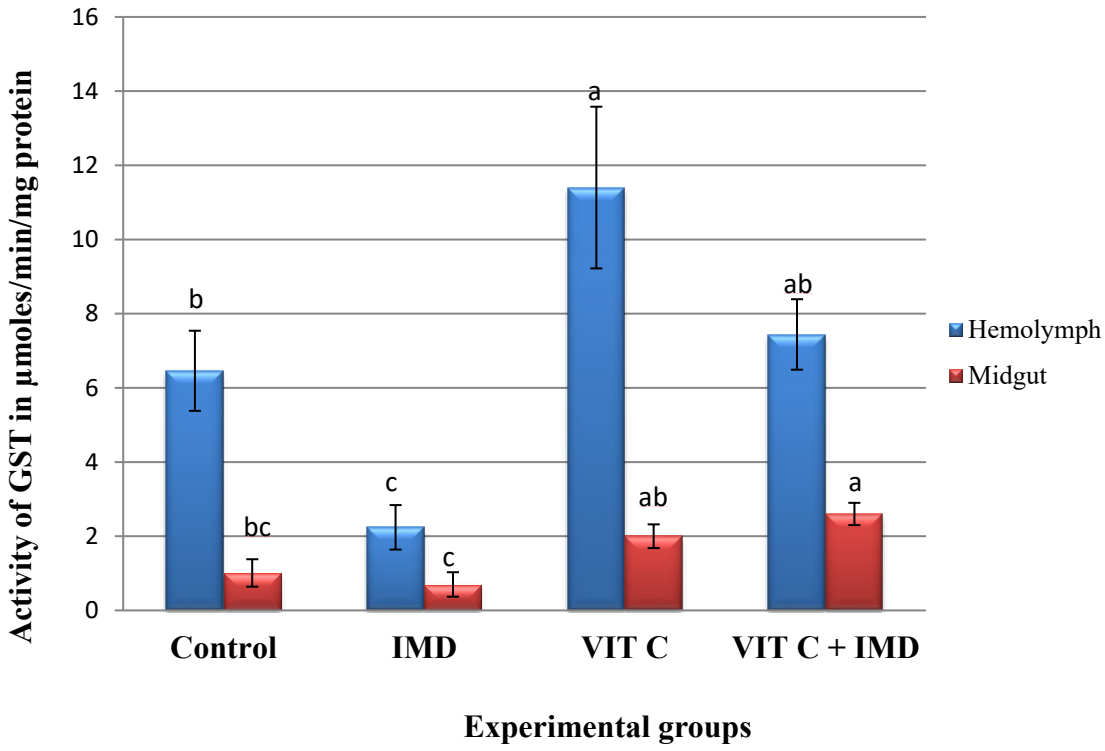


Table 4.9. The activity of GST in the hemolymph and mid gut of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Hemolymph	6.45 ± 2.6^{b}	2.24 ± 1.4^{c}	11.4 ± 5.3^{a}	7.44 ± 2.3^{ab}
Midgut	1.01 ± 0.3^{bc}	0.7 ± 0.3^{c}	2.0 ± 0.3^{ab}	2.6 ± 0.7^{a}

4.3.5. Activity of Glutathione peroxidase (GPx)

The activity of the enzyme Glutathione peroxidase was measured in the hemolymph and midgut of worker bees (Figure 4.10). The activity of this enzyme in the hemolymph was significantly higher ($p \leq 0.05$) in the treatments with IMD (467.8±58.3), supplementation with VIT C (514.3±115.2) and VIT C+IMD (545.3±78.7) compared to the control (233.8±13.2). In midgut tissue, exposure to IMD reduced the activity of the enzyme (287.2±91.8). However, supplementation of the diet with VIT C alone (475.4±63.7) or in combination with exposure to IMD (511.5±37.5) significantly increased enzyme activity in the midgut (Table 4.10).

Figure 4.10. Specific activity of GPx in the hemolymph and midgut tissues of worker bees.

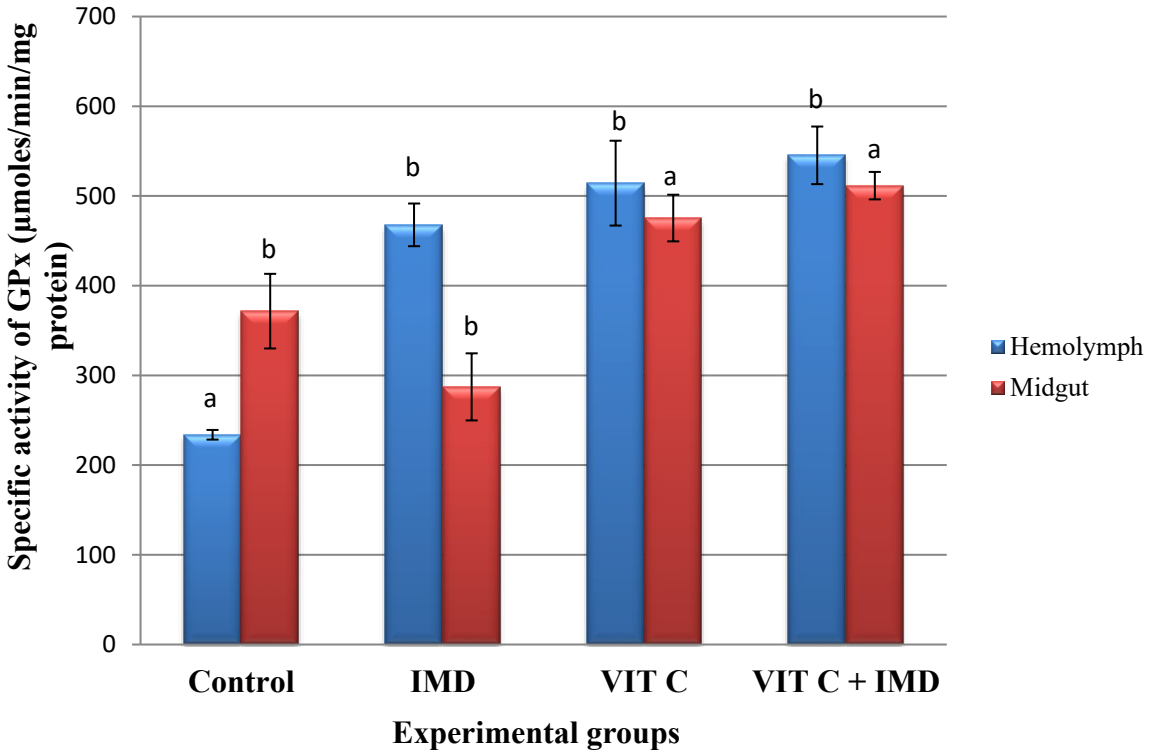


Table 4.10. The activity of GPx in the hemolymph and mid gut of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Hemolymph	233.8±13.2^{a}	467.8±58.3^{b}	514.3±115.2^{b}	545.3±78.7^{b}
Midgut	371.6±101^{b}	287.2±91.8^{b}	475.4±63.7^{a}	511.5±37.5^{a}

4.3.6. Activity of Glutathione Reductase (GSR)

GSR activity was measured in the hemolymph and midgut of worker bees (Figure 4.11). It was found that the activity of the enzyme was reduced in the hemolymph (0.11±0.04) and midgut (0.27±0.21) when worker bees were exposed to IMD. Statistical analysis showed a significant increase in GSR activity in the hemolymph of the group supplemented with VIT C (0.37±0.11) and the VIT C + IMD group (0.76±0.23) compared with the control (0.16±0.05). In the group exposed to VIT C + IMD, the activity of GSR showed a significant increase in the hemolymph, while its activity in the midgut was equal to that of the control (0.47±0.45) (Table 4.11).

Figure 4.11. Specific activity of GSR in the hemolymph and mid gut tissues of worker bees.

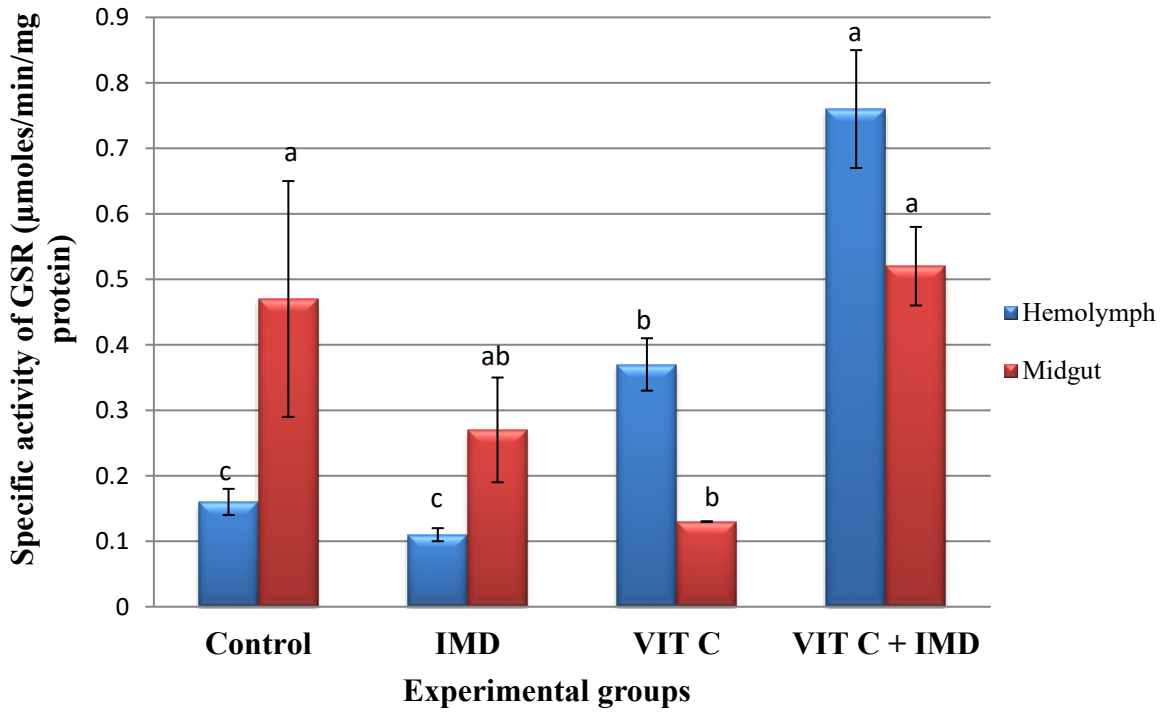


Table 4.11. The activity of GSR in the hemolymph and mid gut of worker bees.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
Hemolymph	0.16 ± 0.05^{c}	0.11 ± 0.04^{c}	0.37 ± 0.11^{b}	0.76 ± 0.23^{a}
Midgut	0.47 ± 0.45^{a}	0.27 ± 0.21^{ab}	0.13 ± 0.02^{b}	0.52 ± 0.15^{a}

4.4. Expression of nicotinic acetylcholine receptors

Expression of nAChRs subunits in the brain of worker bees was studied (Table 4.12). Administration of IMD as a single agent significantly increased the expression of *nAChRα1* and *nAChRα2* subunits. The transcript abundance of *nAChRα1* and *nAChRα2* strongly increased upon exposure to imidacloprid. Transcripts of *nAChRα1* showed a 4.7-fold increase over control when exposed to imidacloprid alone, whereas transcripts of *nAChRα1* in the VIT C group showed a 1.3-fold increase. In the VIT C + IMD group, the transcripts of *nAChRα1* showed a 2.3-fold increase compared with the control group (Figure 4.13). The *nAChRα1* levels in the IMD-exposed group were significantly different from the control group (control: 32.25 ± 1.7, IMD: 26.75 ± 1.7; mean ± standard deviation), whereas the *nAChRα1* levels in the VIT C (30.0 ± 1.8) and VIT C + IMD (29.75 ± 2.2) groups were not significantly different from the control group (Figure 4.12).

Figure 4.12. Relative mRNA expression of *nAChRα1* in the brain of honey bees.

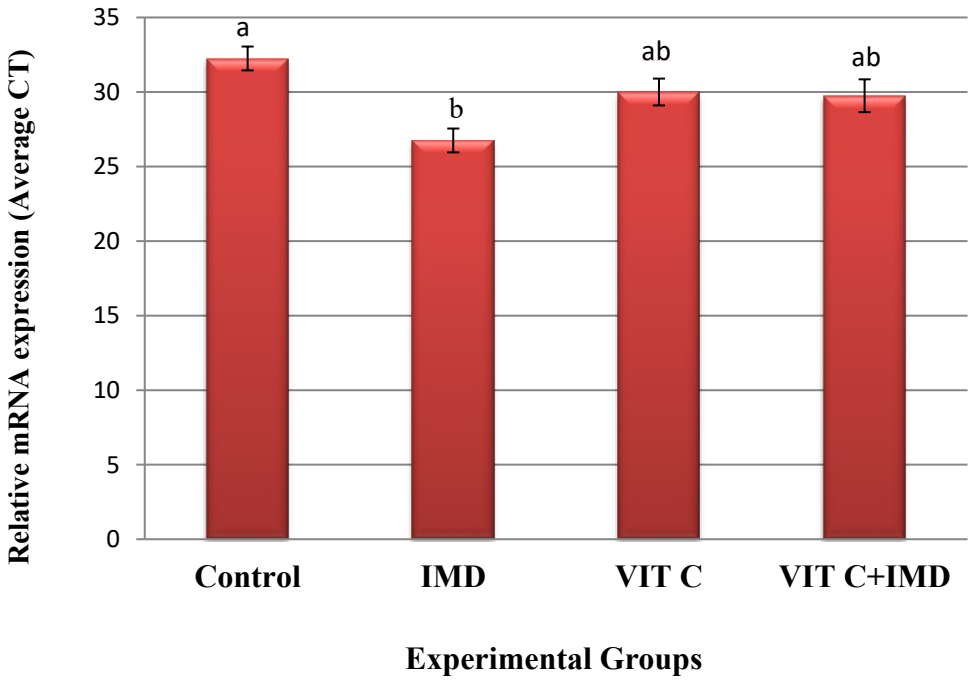


Figure 4.13. The abundance of *nAChRα1* transcripts in the brain of honey bees.

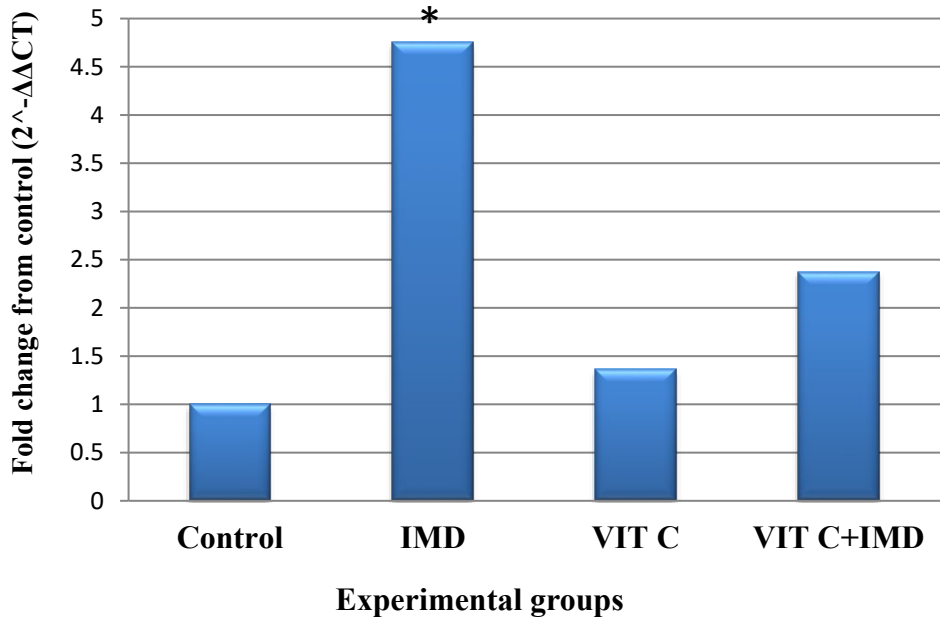


Table 4.12. The Relative mRNA expression of the nicotinic acetylcholine receptor subunits *nAChRα1* and *nAChRα2.*

Experimental groups	Control	IMD	VIT C	VIT C + IMD
nAchrα1	32.25±1.7^{a}	26.75±1.7^{b}	30.0±1.8^{ab}	29.75±2.2^{ab}
nAchrα2	33.25±2.3^{a}	26.25±1.7^{b}	32.0±2.1^{a}	30.25±1.7^{ab}

The transcripts of *nAChRα2* showed similar expression profiles upon exposure to imidacloprid. Transcripts of *nAChRα2* showed a 3.7-fold increase over control when exposed to imidacloprid alone, whereas transcripts of *nAChRα2* in the VIT C group showed a 1.1-fold increase. In the VIT C + IMD group, the transcripts of *nAChRα2* showed a 2.2-fold increase compared with the control group (Figure 4.15). The *nAChRα2* levels in the IMD-exposed group were significantly different from the control group (control: 33.25 ± 2.3, IMD: 26.25 ± 1.7; mean ± standard deviation), whereas the *nAChRα2* levels in the VIT C (32.0 ± 2.1) and VIT C + IMD (30.25 ± 1.7) groups were not significantly different from the control group

(Figure 4.14). In the VIT C + IMD group, the transcripts of *nAChRα1* and *nAChRα2* showed a similar expression pattern. In this group, the transcripts of *nAChRα1* and *nAChRα2* were decreased compared with the IMD-exposed group.

Figure 4.14. Relative mRNA expression of *nAChRα2* in the brain of honey bees.

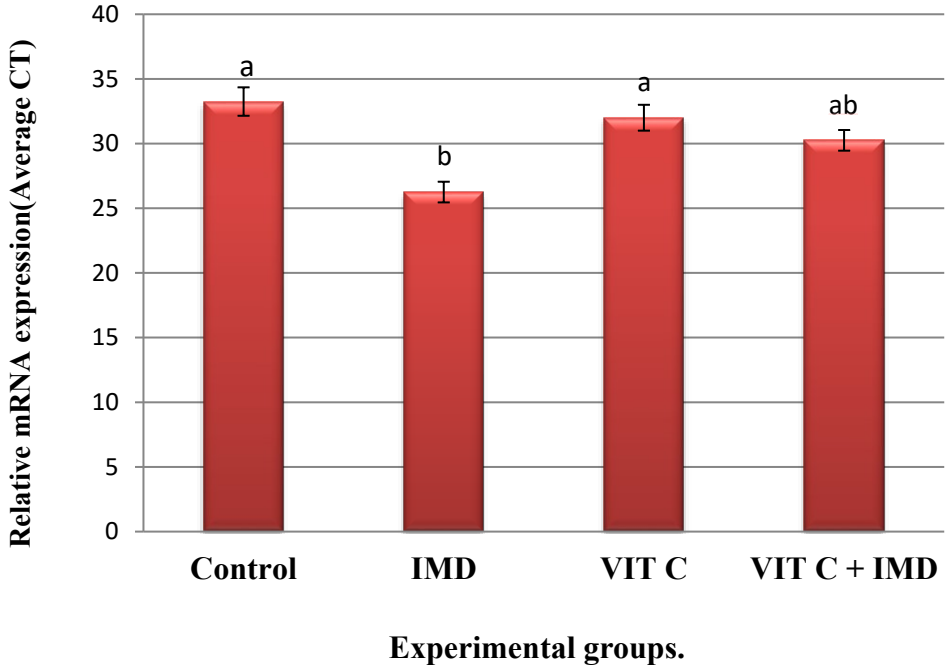
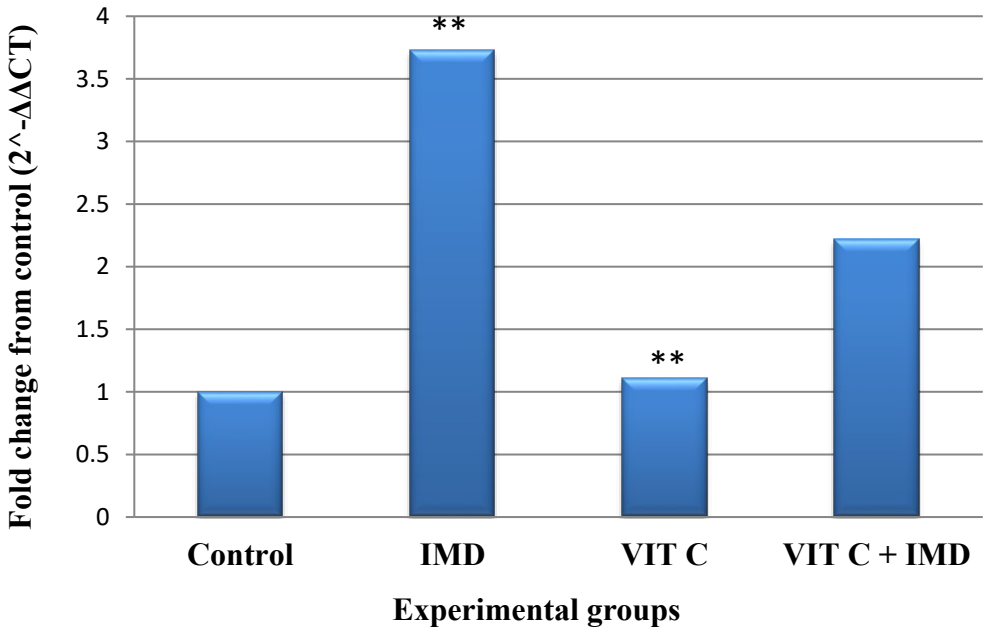


Figure 4.15. The abundance of *nAChRα2* transcripts in the brain of honey bees.



4.5. Expression of Vitellogenin in the Ovary

The expression of *vitellogenin* in the ovaries of queen bees was studied. *Vitellogenin* was found to be down-regulated in the ovaries of queen bees upon exposure to imidacloprid (0.38-fold decrease compared to control) (Figure 4.17), while the transcripts of *vitellogenin* were increased 1.1-fold in the VIT C group compared to control. In the VIT C + IMD group, *vitellogenin* transcripts showed a similar expression profile as in the control group (1.02-fold difference compared with the control). *Vitellogenin* levels in the IMD-exposed group were not significantly different from control (control: 30.5 ± 1.2, IMD: 33.5 ± 1.7; mean ± standard deviation). No significant difference was observed in VIT C + IMD (31.0 ± 1.6) compared with control. In contrast, *vitellogenin* levels in the IMD group were significantly different from those in the VIT C group (29.25 ± 0.9) (Figure 4.16).

Figure 4.16. Relative mRNA expression of *Vitellogenin* in the ovary of queen bees.

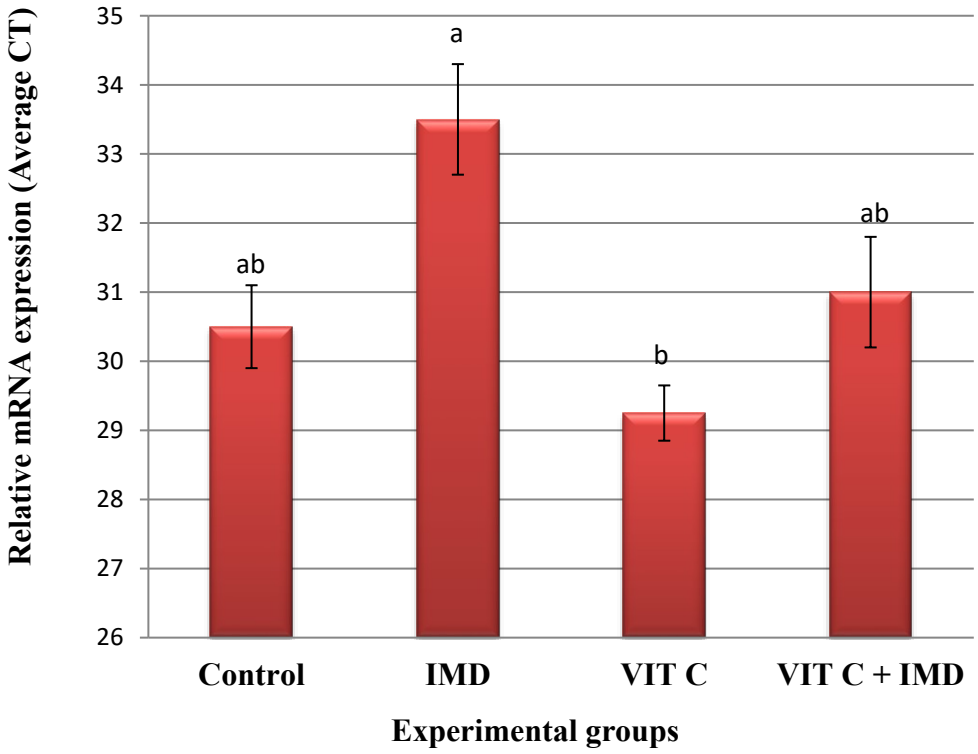


Figure 4.17. The abundance of *Vitellogenin* transcripts in the ovary of queen bees.

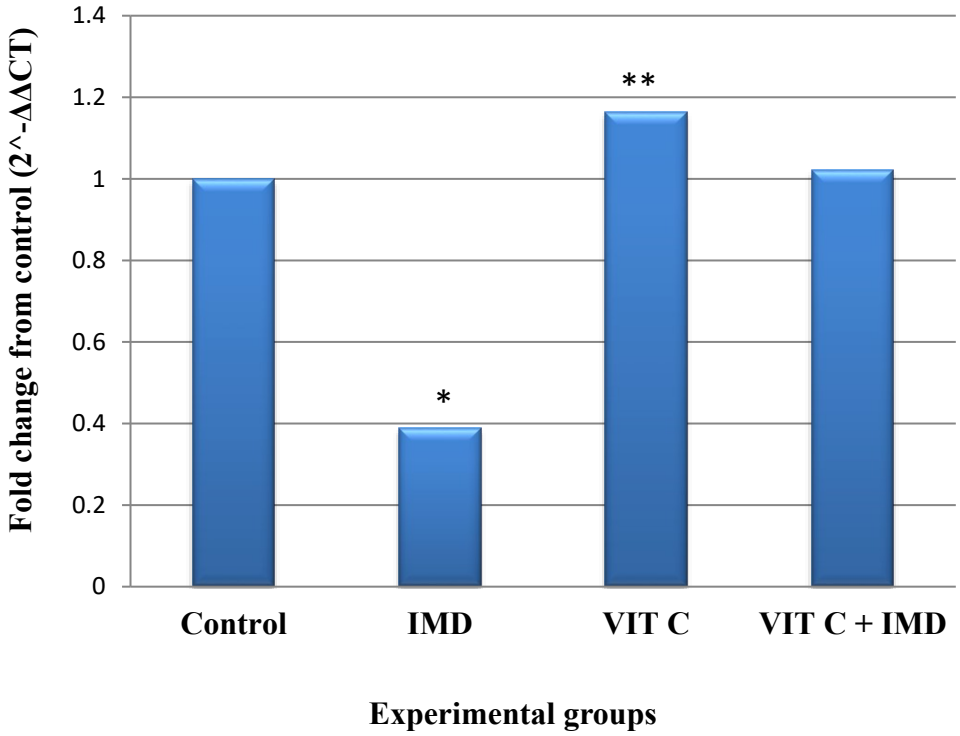


Table 4.13. The Relative mRNA expression of *vitellogenin* and *CYP336A1* in the ovary.

Experimental groups	Control	IMD	VIT C	VIT C + IMD
vitellogenin	30.5±1.2^{ab}	33.5±1.7^{a}	29.2±0.9^{b}	31.0±1.6^{ab}
CYP336A1	32.0±1.8^{ab}	34.2±0.9^{a}	30.0±1.4^{b}	31.7±0.9^{ab}

4.6. Expression of CYP336A1 in the Ovary

In the ovaries of queen bees, the transcripts of *CYP336A1* and *vitellogenin* showed a similar expression profile (Table 4.13). *CYP336A1* was found to be down-regulated in the ovaries of queen bees upon exposure to imidacloprid (0.2-fold decrease compared to control) (Figure 4.19), while the transcripts of *CYP336A1* in the VIT C group showed a 2.1-fold increase compared to control. In the VIT C + IMD group, *CYP336A1* transcripts showed a similar

expression profile as in the control group (1.1-fold difference compared with the control). *CYP336A1* levels in the IMD-exposed group were not significantly different from control (control: 32.0 ± 1.8, IMD: 34.25 ± 0.9). No significant difference was observed in VIT C + IMD (31.75 ± 0.9) compared with control. In contrast, *CYP336A1* levels in the IMD group were significantly different from those in the VIT C group (30.0 ± 1.4) (Figure 4.18).

Figure 4.18. Relative mRNA expression of *CYP336A1* in the ovary of queen bees.

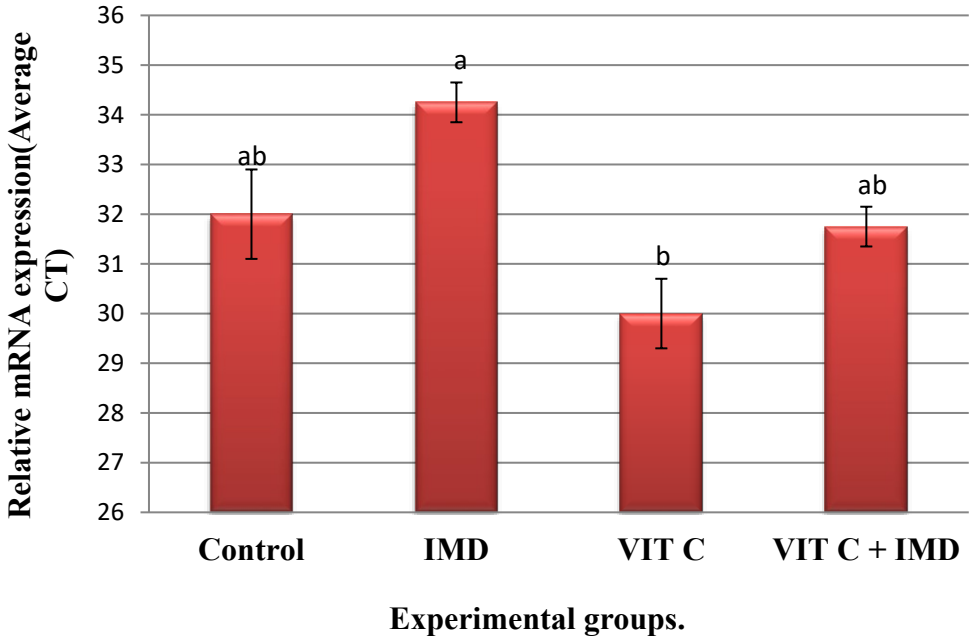
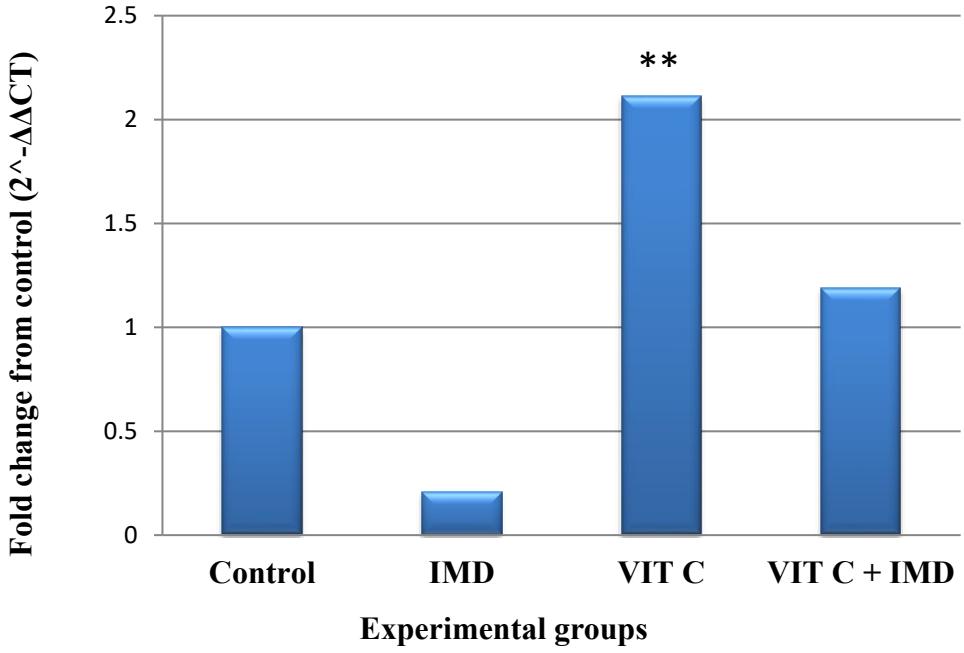


Figure 4.19. The abundance of *CYP336A1* transcripts in the ovary of queen bees.



4.7. Histological alterations in the Ovary

The vitellarium with oocyte and accompanying trophocyte from the ovary of the control group is shown in (Plate 1. A). In the control group, the oocyte and trophocyte are connected by a trophic stalk. The oocyte is surrounded by a single layer cuboidal epithelium (Plate 2. A). The trophocyte is dense with granular cytoplasm and a large polymorphic nucleus. Germarium with germline cells was found to be normal in the control group.

Histological analysis revealed degeneration of oocytes and trophocytes characterized by cytoplasmic vacuolization and increasing intercellular spaces in ovarioles upon exposure to IMD (Plate 2. B). When IMD was administered to queen bees, ovaries showed abnormal histology, such as perinuclear vacuolization and pyknotic nuclei in trophocytes, indicating early degeneration of trophocytes. In the vitellarium, cytoplasmic vacuolization followed by oocyte degeneration was observed (Plate 1. B). Oocyte shrinkage and intercellular spaces were also observed in the cuboidal epithelium.

Histological analysis of the VIT-C group showed a vitellarium with oocyte and accompanying trophocytes in a normal pattern (Plate 1. C). The trophocytes are dense with granular cytoplasm and a large polymorphic nucleus. The oocytes are surrounded by a single layer of cuboidal epithelium that weakens in the later stages of vitellogenesis (Plate 2. C).

Histological examination of the queen ovary from the VIT C + IMD group revealed perinuclear vacuolization and pyknotic nuclei in the trophocytes (Plate 1. D). In contrast, complete degeneration of mature oocytes as in the IMD group was not observed. Perinuclear vacuolization was observed in both the germarium and vitellarium. In the vitellarium, the cuboidal epithelium was not affected and the appearance of intercellular spaces as in the IMD-exposed group was not observed in the VIT C + IMD group (Plate 2. D).

4.8. Histological alterations in the hypopharyngeal gland

Histological analysis of hypopharyngeal glands of worker bees from the control group showed round shaped acini with numerous secretory vesicles. Each acinus consisted of an irregular nucleus surrounded by secretory vesicles. Each secretory cell consisted of 8-10 secretory vesicles of different sizes. The extracellular spaces between adjacent acinar cells were narrow and thin compared to the other groups (Plate 3. A).

The hypopharyngeal glands of worker bees exhibited cytoplasmic vacuolization in the acini and malformations in their shape when exposed to IMD. Increased numbers of secretory vesicles and fusion and lysis of secretory vesicles were observed in the secretory cells (Plate 3. B).

The hypopharyngeal glands in the VIT-C group consisted of large acini compared with the control group. The acini have a round shape with clusters of multiple secretory units. The secretory cells consist of 10-12 secretory vesicles of different sizes, and the extracellular space between two adjacent acini is wider than in the control group (Plate 3. C).

In the VIT C + IMD group, the hypopharyngeal gland had malformed small acini and normal large acini compared with the control group and the VIT C group. The large acini consisted of clusters of multiple secretory units and 10-12 secretory vesicles of different sizes. Coalescence and lysis of secretory vesicles were not observed in this group, as was the case in the IMD-exposed group (Plate 3. D).

4.9. Histological alterations associated with the midgut

4.9.1. Mid gut of worker bees

Histological analysis of the midgut of honey bees from the control group showed a typical morphology of the epithelium with a thin gelatinous layer. In some regions the midgut epithelium was higher and in some places it is not well defined. At the base of the epithelium, the nuclei are surrounded by lighter areas (hallos) with vacuolated cell cytoplasm. The pollen grains are surrounded by numerous layers of peritrophic membrane. In some locations, tearing of the peritrophic membrane from the intestinal epithelium was clearly visible. The intestinal lumen is wide in the control group (Plate 4. A).

Morphological assessment of worker bees exposed to imidacloprid (IMD) showed adverse effects where the peritrophic membrane was completely ruptured and the midgut contents were distributed in the lumen. Degenerated epithelial cells with loss of the gelatinous matrix and large vacuoles were observed (Plate 4. B).

The VITC group showed a well-defined epithelium with a thick gelatinous layer. The nuclei were covered with hallos and the cell cytoplasm was less vacuolated. The epithelium did not have well-defined folds and was thicker in some regions. The intestinal contents were covered with a multilayer peritrophic membrane. Tearing of the peritrophic membrane was

observed in some regions. The intestinal lumen was comparatively wider in this group than in the control group (Plate 4. C).

Adverse effects were less severe in the VIT C + IMD group than in the IMD-exposed group. The peritrophic membrane was not affected and appeared normal with multiple layers. The thickness of the epithelial layer was the same as in the VIT C group. An abnormal increase in the number of large vacuoles was observed in this group compared with the VIT C and control groups. Degeneration of epithelial cells and loss of gelatinous matrix were not observed as in the IMD-exposed group (Plate 4. D).

4.9.2. Mid gut of queen bees

Histological analysis of the midgut of queen bees from the control group revealed a single epithelial layer with less conspicuous epithelial folds. The peritrophic membrane appeared as stratified amorphous material. Proliferation of digestive cells from the epithelial folds was clearly visible (Plate 5. A). The peritrophic membrane was completely ruptured and scattered in the lumen when exposed to IMD. The epithelial layer showed vacuolization and degenerated basement membrane in the IMD group compared to the control group (Plate 5. B).

Histological analysis of the midgut of queen bees from the VIT C group showed a well-structured thick epithelium with epithelial folds. The peritrophic membrane was not found as a layered structure, but appeared as a thick layer of amorphous material (Plate 5. C). In the VIT C + IMD group, the adverse effects were less severe. It was found that the epithelial cells were not completely degenerated and the basement membrane was not completely ruptured compared with the IMD-exposed groups. An increase in the number of enterocytes and the presence of large vacuoles were also observed in this group (Plate 5. D).

Plate 1. Photomicrographs of the Ovary of *A. cerana indica* stained with H&E. **A-D** includes, **A**. Ovary of the queen bees from the control group **B**. Ovary of the queen bees exposed to imidacloprid. **C**. Ovary of the queen bees from the ascorbic acid supplemented group. **D**. Ovary of the queen bees from the ascorbic acid supplemented group exposed to imidacloprid. O- oocyte, T- trophocyte, n-nucleus, V-vacuole, C- cuboidal epithelium.

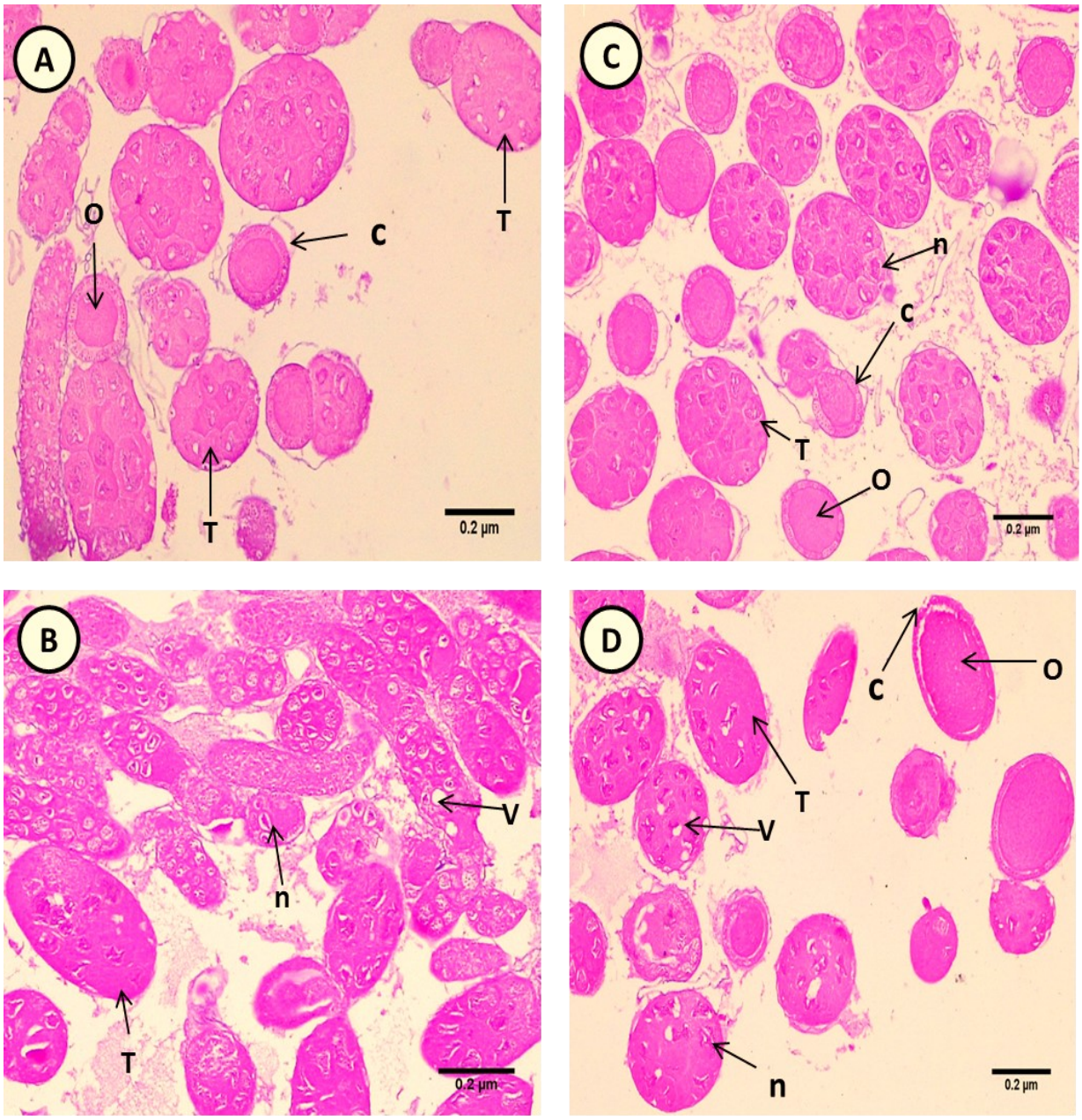


Plate 2. Photomicrographs of the oocyte of the Queen bees (***A. cerana indica***) stained with H&E. **A-D** includes, **A**. Ovary of the queen bees from the control group showing oocyte and cuboidal epithelium. **B**. Ovary of the queen bees exposed to imidacloprid showing shrinkage of oocyte and intercellular spaces in the cuboidal epithelium. **C**. Ovary of the queen bees from the ascorbic acid supplemented group showing oocyte and cuboidal epithelium. **D**. Ovary of the queen bees from the ascorbic acid supplemented group exposed to imidacloprid showing oocyte surrounded with cuboidal epithelium. O- oocyte, T- trophocyte, C- cuboidal epithelium.

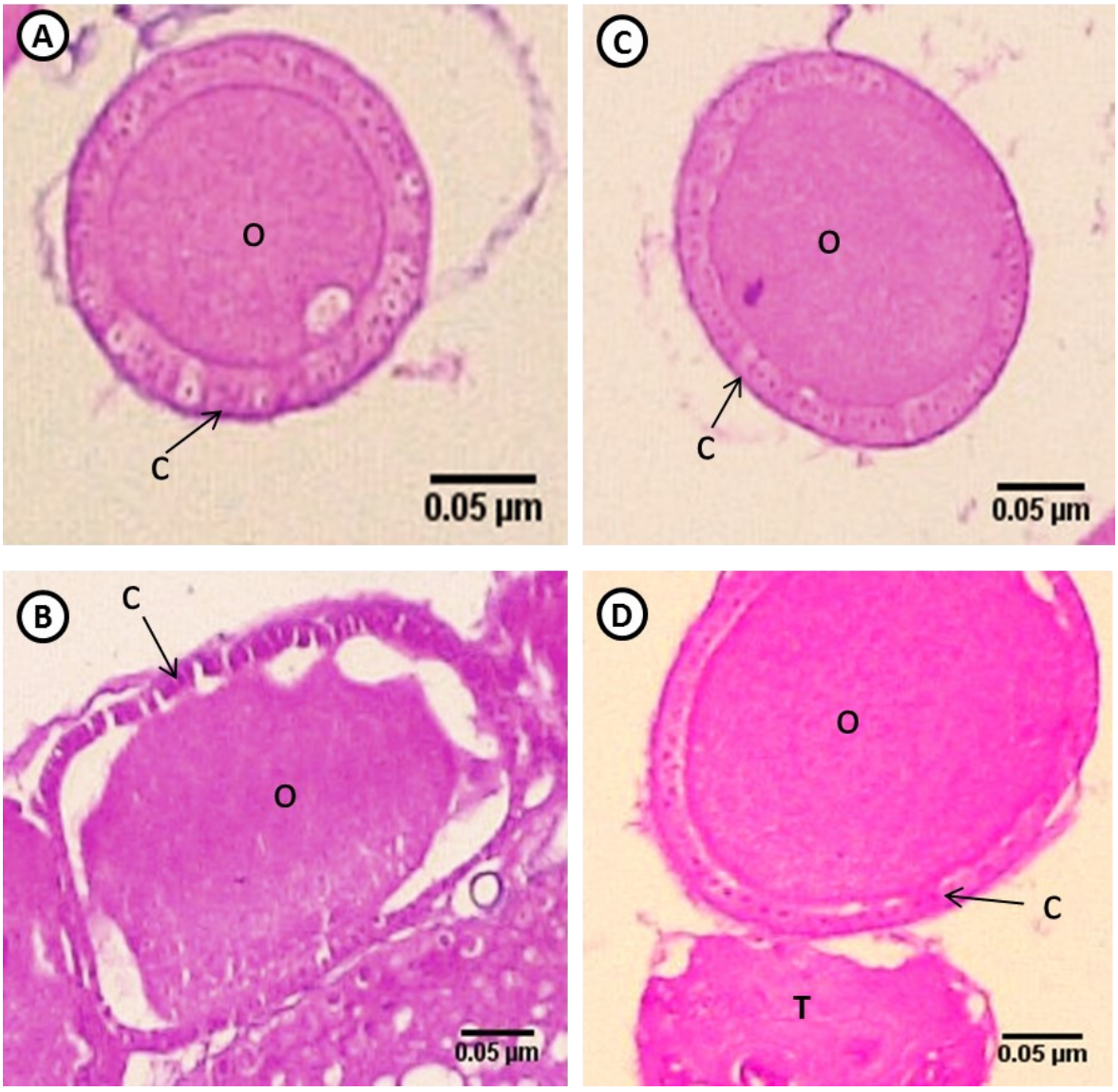


Plate 3. Photomicrographs of the hypopharyngeal glands of worker bees (***A. cerana indica***) stained with H&E. **A-D** includes, **A**. Hypopharyngeal glands of worker bees from control group **B**. Hypopharyngeal gland of worker bees on exposure to imidacloprid. **C**. Hypopharyngeal glands of worker bees from the ascorbic acid supplemented group. **D**. Hypopharyngeal glands of ascorbic acid supplemented worker bees exposed to imidacloprid. **ac**- acini, **n**- nucleus, **sv**-secretory vesicles, **ex**- extracellular space, **D**-main duct, Asterisks indicates merging and lysis of cells.

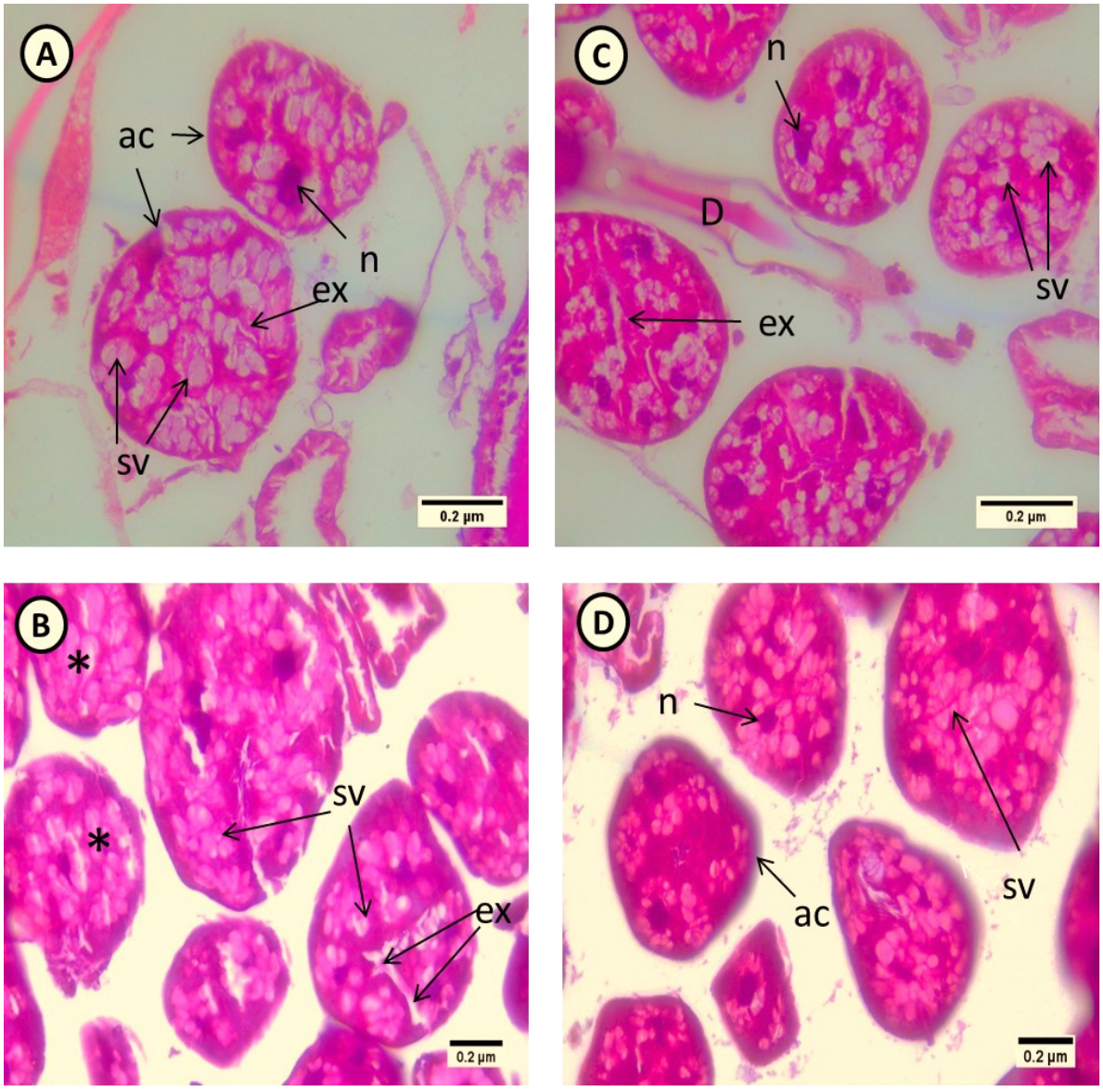


Plate 4. Photomicrographs of the midgut cells of worker honeybee (***A. cerana indica***) stained with H&E. **A-D** includes, **A**. midgut cells of honeybee workers from the control group. **B**. midgut cells of honeybee workers exposed to **IMD**. **C**. midgut cells of honeybee workers supplemented with ascorbic acid. **D**. midgut cells of ascorbic acid supplemented worker bees exposed to imidacloprid. **E-** Epithelium; **PM**-Peritrophic Membrane ; **L-** Lumen.

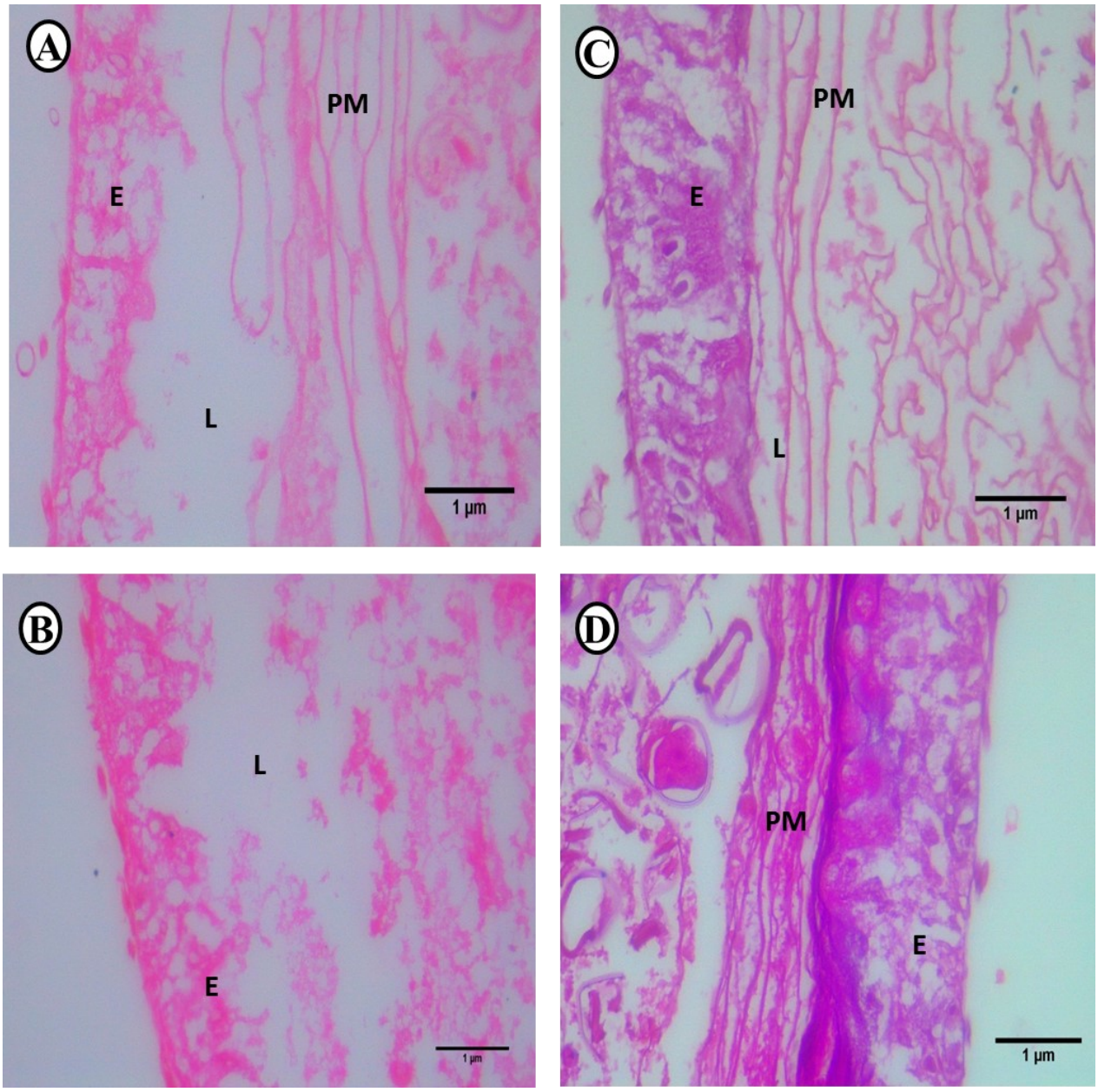
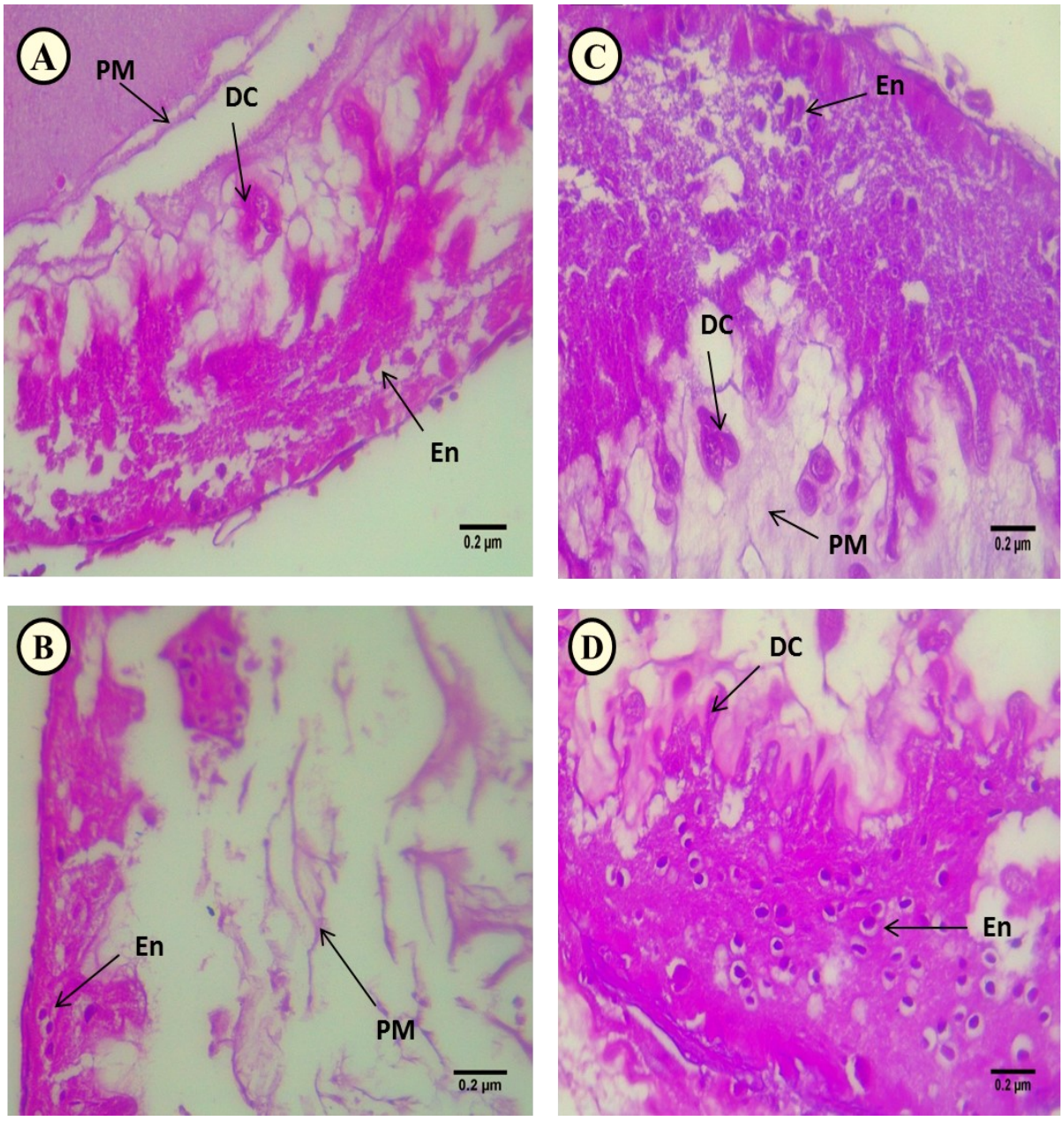


Plate 5. Photomicrographs of the midgut cells of Queen bees (***A. cerana indica***) stained with H&E. **A-D** includes, **A**. midgut cells of queen bees received sugar syrup **B**. midgut cells of queen bees exposed to **IMD**. **C**. midgut cells of queen bees supplemented with ascorbic acid. **D**. midgut cells of ascorbic acid supplemented queen bees exposed to imidacloprid. **PM**- Peritrophic Membrane, **DC**- Proliferating digestive cells, **En**- Enterocytes.



Chapter 5.
Discussion

Contents

Chapter 5. Discussion

5.1. Acute toxicity of Imidacloprid

Pesticides have long been used in agriculture to control insect infestations in order to increase the quantity and quality of food. As the use of pesticides increased, the concerns about their negative effects on non-target organisms also increased (Ozkara *et al.,* 2016). Neonicotinoids have exceptional potency and systemic activity for crop protection against piercing- sucking pests (Abrol, 2022). Imidacloprid is widely used because of its lower toxicity to non-target organisms, fewer problems with resuscitation and selectivity against pests (Medrzycki *et al.,* 2003).

In this study, the LD_{50} of imidacloprid against the honey bee *A. cerana indica* was 36.03±0.87 µg/mL after 24 hours. The LD_{50} of imidacloprid was reported in honeybees and has been shown to vary among species. According to Vinothkumar *et al.,* (2020), the oral LD50 of imidacloprid was 32.26 ppm and according to Khan and Dethe, (2005), it was 0.0035%. Pastagia and Patel, (2007) observed 80.67% mortality of Indian honey bee *A. cerana* when exposed to 0.05% imidacloprid. According to Suchail *et al.,* (2001), the acute toxicity ($LD_{50)}$ of imidacloprid to honey bees (*Apis mellifera*) is 60 ng/bee (600 g/kg) at 48 hours and about 40 ng/bee (400 g/kg) at 72 and 96 hours. In the field dose, nitro-substituted compounds such as imidacloprid were more hazardous to honey bees (Schmidt, 1996; Iwasa *et al.,* 2004).

The lethal dose/concentration of imidacloprid in honeybees has been studied extensively, and values vary with environmental conditions and season. The oral LD_{50} value is highly dependent on the pesticide and the organism. Sensitivity to insecticides varies among honey bees of different genetic lines (Krainer, Italian, and Russian bee strains), and Italian bees have been found to be most sensitive to neonicotinoids (Rinkevich *et al.,* 2015). Scientific evidence strongly suggests that residue levels of imidacloprid do not lead to immediate death of honey bees, but may have negative effects on the ecological sustainability of honey bees (Pisa *et al.,* 2021). The effects of pesticide exposure are not limited to the individual, but can harm the entire bee colony and even pass to the next generation (Mitchell *et al.,* 2017).

5.2. Effect on biochemical parameters

Toxicological studies evaluating the potential adverse effects of pesticides on bees are critical because they enable the development of initiatives to protect and conserve pollinators. The effects of a sublethal dose of imidacloprid and ascorbic acid supplementation on several biochemical parameters were studied in worker bees of *A. cerana indica* . When honey bees were exposed to IMD, protein concentration increased in the hemolymph of worker bees compared to the control group. Catae *et al.*, (2019) found that worker bees exposed to even trace amounts of imidacloprid triggered a cascade of biochemical changes, including overexpression of a number of proteins involved in critical cellular functions potentially damaged by the insecticide. These biochemical changes may affect colony health and performance. Exposure to imidacloprid resulted in oxidative stress and significant changes in hemolymph proteins in Sydney Rock Oyster (Ewere *et al.,* 2020). The increased total protein content in the hemolymph of worker bees signals the initiation of a detoxification process in which heat shock proteins may be upregulated as part of the cellular stress response, promoting stress tolerance. According to Rand *et al.,* (2015), proteins involved in detoxification, heat shock, antioxidant response, lipid metabolism, amino acid metabolism, and glutathione metabolism were upregulated after nicotine exposure. Their metabolomic and proteomic analyses show active nicotine detoxification in bees associated with increased energy expenditure and antioxidant and heat shock responses.

Synthetic pesticides have been considered an important cause of honey bee population decline, which can negatively affect insect immunity via oxidative stress. Pesticide exposure has been shown to affect both detoxification and immunological responses in honey bees (Schmehl *et al.,* 2014; Goulson *et al.,* 2015). Imidacloprid caused changes in antioxidant enzyme activities, lipid peroxidation levels, biochemical, genotoxic, and immunotoxic effects (Yucel and Kayis, 2019). Enzyme activities have been shown to be suitable indicators for assessing stress responses in honeybees exposed to xenobiotics (Badiou-Beneteau *et al.,* 2012; Badawy *et al.,* 2015). To investigate metabolic detoxification, we used acetycholinesterase (AChE) and glucose-6-phosphate dehydrogenase (G6PD) to evaluate their involvement in the response to xenobiotics in *A. cerana indica* against imidacloprid.

AChE hydrolyzes acetycholine (Ach), an important neurotransmitter in the brain of insects. The enzyme AChE is important to the nervous system because it terminates nerve impulses by catalyzing the hydrolysis of acetycholine (Shapira *et al.,* 2001). Neonicotinoids bind to the insect nicotinic receptor as agonists of the neurotransmitter acetycholine, resulting in sustained activation of the receptor until the insects die (Grunewald *et al.,* 2019). The effects on AChE can be used to assess the detoxification system responsible for mediating nerve impulse transmission at cholinergic synapses. AChE activity can be used as a potential biomarker of neonicotinoid exposure (Carvalho *et al.,* 2013).

In the present study, it was found that the activity of AChE decreased after exposure to imidacloprid. Similar results were reported in *A. mellifera*, where AChE activity was inhibited after exposure to sublethal concentrations of imidacloprid (Li *et al.,* 2017; Delkash-Roudsari *et al.,* 2022). Similarly, Badawy *et al.,* (2015) found a decrease in specific activity of AChE upon exposure to neonicotinoids. According to Huang *et al.*, (1990), these pesticides or their metabolites can either completely suppress or reduce AChE activity (e.g., by interacting with serine groups outside the catalytic subunit) by binding to nonspecific proteins. Alterations in AChE activity may indicate nervous system dysfunction that can impair memory, olfactory learning, and navigational abilities (Williamson *et al.,* 2013; Gauthier *et al.,* 1992). In addition, changes in AChE activity can lead to developmental problems (Wessler *et al.,* 2016; Kim *et al.,* 2017). In the present study, the VIT C + IMD group showed replenishment of AChE activity, indicating the ability of VIT C to maintain AChE activity against the toxic potential of IMD.

The most important enzyme of the pentose phosphate pathway is glucose-6-phosphate dehydrogenase (G6PD). Although it has been studied by few researchers, this enzyme is rarely used as a biochemical diagnostic for insect pesticide exposure. In the present study, G6PD activity was significantly reduced on exposure to imidacloprid. G6PD is a secondary antioxidant enzyme that produces NADPH, which leads to the regeneration of reduced glutathione, which contributes to the reduction of oxidative stress (Pal *et al.,* 2022) (Figure 5.2). The decrease in G6PD activity suggests that the organism's ability to respond to oxidative stress conditions is insufficient (Aliciguzel *et al.,* 2001). Metabolic pathways such as the TCA cycle, glycolysis and the pentose phosphate pathway (PPP), oxidative

phosphorylation, and fatty acid oxidation have been shown to play a role in immunological responses (Guo *et al.,* 2021). Consequently, any alteration in enzyme activity associated with these metabolic pathways will result in a decreased immunological response. In the present study, supplementation of VIT C increased the activity of G6PD. This may be due to the fact that VIT C effectively scavenges hydrogen peroxide producing NADP (Summers and Felton, 1993). G6PD reduces (NADP) to (NADPH) (Beutler *et al.,* 1994) (Figure 5.3).

In the present study, it was shown that the total carbohydrate content in the hemolymph of worker bees exposed to IMD was lower compared to the control. Carbohydrate content was reduced in *Galleria mellonella* L. with increasing imidacloprid dose (Yucel and Kayis, 2019). Pesticide interactions with malnutrition and disturbance cause energetic stress in honey bees (Schmehl *et al.,* 2014). At low and high concentrations, IMD alter honey bee energy metabolism in several ways, negatively affecting honey bee colony health (Kim *et al.,* 2022). Our results suggest that imidacloprid disrupts honey bee physiology, thereby reducing the efficiency of this beneficial pollinator.

Exposure to pesticides has been shown to have a significant effect on the expression of genes involved in detoxification, behavioral maturation, immunity, and nutrition in worker bees. Because the responses to pesticides and nutrition are so similar, providing honey bees and other pollinators with high-quality nutrition may help them resist the effects of pesticides (Schmehl *et al.,* 2014). According to Farjan *et al.,* (2012), vitamin C can be recommended as a natural, safe, and very cost-effective dietary supplement to increase resistance to stress effects, including oxidative stress. The present result supports these data, in which ascorbic acid supplementation increased the activities of G6PD and AChE. The main function of ascorbic acid is to neutralize free radicals, and because it is water-soluble, it can combat free radical damage both inside and outside cells. Ascorbic acid is a good electron donor; therefore, it can donate electrons to free radicals such as hydroxyl and superoxide radicals and reduce their reactivity (Bendich *et al.,* 1986).

The increased activities of G6PD and AChE in the present study indicated the protective role of ascorbic acid against imidacloprid-induced oxidative stress. Ascorbic acid protects against neonicotinoid-induced systemic toxicity by suppressing ROS signaling pathways, apoptosis, and lipid peroxidation (Xu *et al.,* 2022). As a free radical scavenger,

ascorbic acid can directly and rapidly remove free radicals, thus exerting an antioxidant function. According to the results of this study, ascorbic acid has a beneficial effect on the total carbohydrate content of bees. These results are in agreement with the findings of Farjan *et al.,* (2015) who observed positive effects of ascorbic acid on carbohydrate metabolism. The results of this study showed that a diet enriched with the antioxidant ascorbic acid protects honey bees from oxidative stress caused by imidacloprid. Adequate supply of ascorbic acid to honey bees that are regularly exposed to these pesticides is therefore an effective way to counteract the negative effects of imidacloprid.

5.3. Effect on the antioxidative system

The present study provides new evidence on the ability of dietary ascorbic acid supplementation to reduce peroxidative damage caused by imidacloprid. The specific antioxidant enzyme content in the honeybee *A. cerana indica* was investigated to understand the tolerance and detoxification strategies against IMD toxicity. The present observations showed that exposure of worker honeybees to IMD had significant effects on the activities of antioxidant enzymes (Figure 5.1)

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are key components in maintaining redox balance, as they can scavenge excess free radicals and be the first line of defense against various types of such oxidative radicals. SOD and CAT are the enzymes that act as subsequent components in the antioxidant reaction system of cells. CAT converts superoxide (O_2^-) into H_2O_2 and SOD converts the H_2O_2 thus formed into H_2O and O_2. Various enzymatic radical scavengers such as catalase, glutathione peroxidase, glutathione S-transferase, peroxidase, superoxide dismutase have been shown to be present in bees (Weirich et al., 2002; Strachecka *et al.,* 2014; Strachecka *et al.,* 2017). Increased levels of antioxidant enzymes may therefore be an important indication of an organism's efforts to counteract the pesticide induced oxidative stress.

The primary function of catalase enzyme is the conversion of hydrogen peroxide (H_2O_2) into H_2 and O_2. H_2O_2 is one of the most stable reactive oxygen species. Catalase is an antioxidant enzyme found in many cell types and is constituted by several sulf- hydryl groups (mainly of the amino acid, cysteine) in its internal structure. Neutralization of free radicals by

this antioxidant is through the expenditure of its sulf- hydryl groups (Deisseroth and Dounce, 1970; Chance *et al*., 1979; Egaas *et al*., 1999; Milton, 2004; Enayati *et al*., 2005; Kirkman and Gaetani, 2007). We observed a significant reduction in the activity of catalase enzyme in both the midgut and hemolymph regions of IMD alone administered worker bees.

Down regulation of catalase activity is coupled with augmented defenselessness to oxidative stress. Uncontrolled free radical production may be due to the saturation of the available sulf- hydryl groups of catalase enzyme. This imbalance in the redox status may in turn attack the catalase enzyme and can cause denaturation of this protein structure. The overall result is the damage to the cell membranes by H_2O_2 induced oxidative damages (Goth *et al*., 2004; Ho *et al*., 2004). Ascorbic acid as an adjuvant, significantly elevate the levels of catalase enzyme in both the midgut and hemolymph regions of worker bees. The protective potential of this vitamin is supposed to be due to its capability to reduce the free radical induced damages by either reducing the levels of free radicals or by stimulating the activity of catalase enzyme.

Superoxide dismutase refers to a group of metalloenzymes that serve as the front line defense mechanism against tissue injuries due to reactive oxygen species (ROS). These proteins catalyze the dismutation of superoxide anion free radical (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2) and thereby decreasing O_2^- levels which otherwise can damage the cells at excessive concentrations (Younus, 2018). Our study showed a reduction in activity of SOD in the hemolymph and midgut tissues on exposure to IMD. The reduced activity of the scavenging enzymes observed in the study reflects the excess ROS generation due to the exposure of IMD. Our findings are in line with Kapoor *et al*., (2010). On the other hand, ascorbic acid supplementation was found to enhance the activity of SOD in the corresponding group. The elevated level of SOD is supposed to be due to the boosting of dismutation process which in turn can facilitate the elimination of ROS.

The major purpose of the peroxidase enzyme is the breakdown of H_2O_2 to nontoxic components (Thangudu and Su, 2021). Significant elevation in POD levels was observed in IMD alone administered groups as observed in our study. This elevation in peroxidase levels is an indication of the enhanced synthesis of peroxidase enzyme to counter the increased volume of free radicals in the tissues. Ascorbic acid as an adjuvant was found to cause a

significant reduction in peroxidase enzyme levels and this probably indicates the capability of this vitamin in alleviating the stress generated by oxidative species.

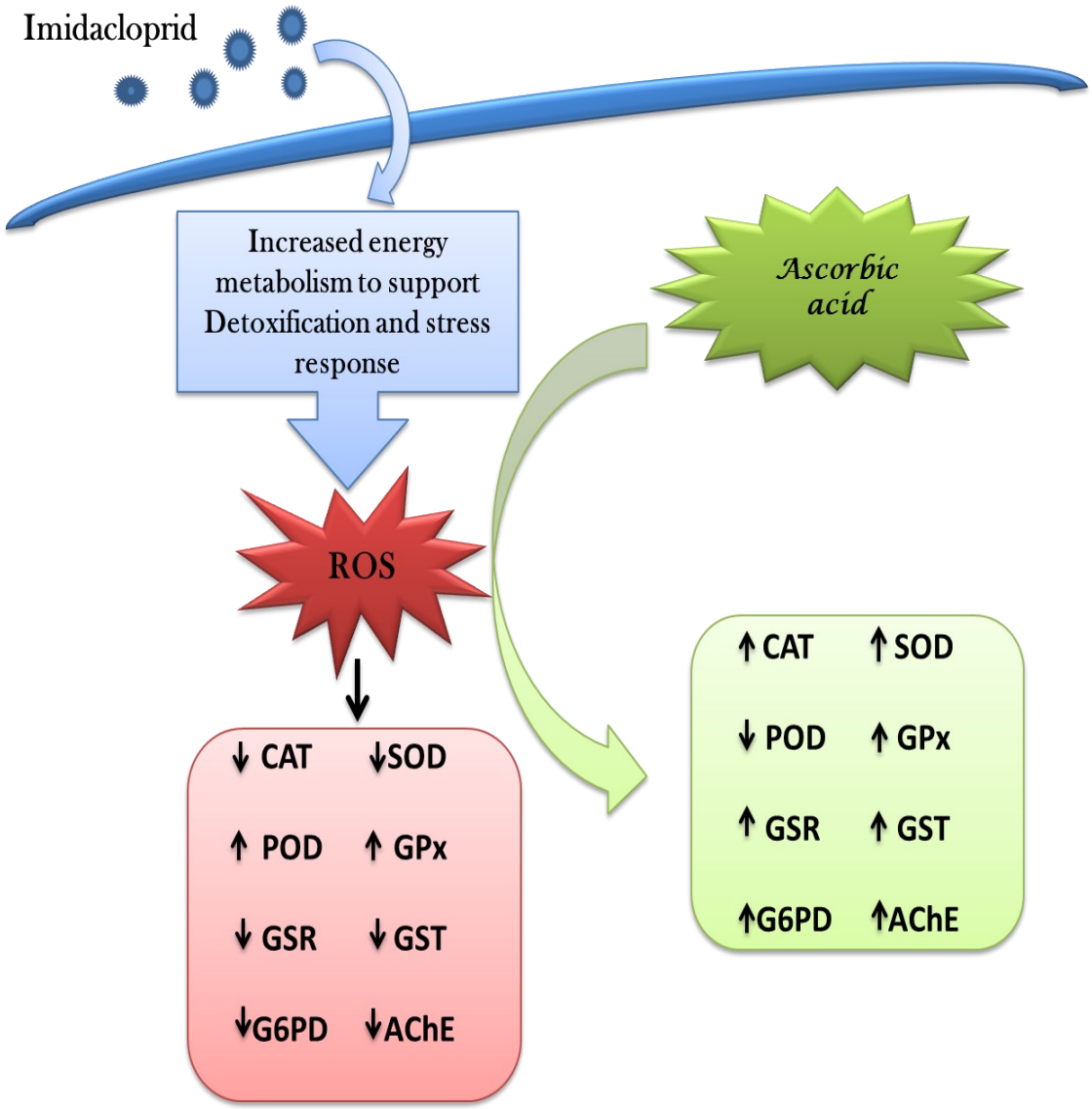


Figure 5.1. Schematic overview of the effect of ascorbic acid supplementation on oxidative stress and associated changes in enzyme activities upon exposure to imidacloprid.

↓ - decrease in enzyme activity, ↑ - increase in enzyme activity, CAT- Catalase, SOD- Superoxide Dismutase, POD- Peroxidase, GPx- Glutathione Peroxidase, GSR- Glutathione Reductase, GST- Glutathione S Transferase, G6PD- Glucose 6 Phosphate Dehydrogenase, AChE- Acetylcholinesterase.

Glutathione S-transferase belongs to a family of detoxification enzymes phase II, whose function is to protect various cellular macromolecules from attack by reactive electrophiles. GST is a multifunctional enzyme involved in oxidative stress protection and may also contribute to phase I detoxification (Rand *et al.,* 2015). GSTs regulate the levels of an enormous range of electrophiles by conjugating them with glutathione (GSH). The conjugation of glutathione is the most important step in the mercapturic acid pathway, facilitating the elimination of toxic compounds (Townsend and Tew, 2003). Honey bees are highly sensitive to certain insecticides, as evidenced by the complete absence of epsilon-class GST and the presence of a single delta-class GST (Claudianos *et al.,* 2006).

The observations from our study indicate a significant decrease in GST content in the hemolymph and midgut tissues of worker bees exposed to IMD. This could be due to the increased utilization of GSH in neutralizing the larger amounts of electrophilic toxic compounds produced by IMD administration. The decrease in GST activity could be explained by the absence of conjugation with GST, synthesis of GST conjugates at undetectable levels, or the drastic down-regulation of GST by imidacloprid. Therefore, a decrease in GST activity could be a sign of honeybees' declining ability to detoxify these pesticides and to combat oxidative stress resulting from exposure to imidacloprid.

Ascorbic acid as an adjuvant showed a different scenario with a significant increase in GST levels in the respective groups. The increase in GST activity may be due to the fact that ascorbic acid decreases the level of oxidative radicals, which in turn leads to a lower consumption of GSH. This increased GSH concentration may provide protection against cellular stress by scavenging excess free radicals. According to Farjan *et al.,* (2012), ascorbic acid supplementation resulted in higher glutathione levels and enhanced GST activity. Increased levels of GST activity have been associated with insecticide resistance (Enayati *et al.,* 2005). Detoxification by GST occurs by passive binding to insecticides or by removal from ROS and detoxification of the products of lipid peroxidation. Glutathione S-transferases are involved in detoxification of DDT by dehydrochlorination, with glutathione acting as a co-factor (Che-Mendoza *et al.,* 2009; Clark and Shamaan, 1984).

Glutathione peroxidase refers to a family of enzymes with peroxidase activity that has the biological task of protecting organisms from oxidative damage. The main function of

GPx is the reduction of lipid hydroperoxides to their secondary alcohols and the reduction of free hydrogen peroxide, regulating the redox balance in the body El-Gendy *et al.,* (2010). In our study, a significant reduction in GPx levels was observed in the midgut of worker bees due to IMD administration. Our observations are in agreement with the results of Kapoor *et al.,* (2010) who also reported a decrease in GPx activity due to IMD exposure. The decreased activity of G6PD due to IMD, as described in the previous section, could be the cause of the decrease in GPx activity. G6PD enables cells to maintain glutathione in its reduced form by reducing NADP to NADPH (Beutler *et al.,* 1994). This in turn allows glutathione peroxidase to eliminate hydrogen peroxide catalyzed by the oxidation of glutathione (GSH) to GSSG, which in turn is reduced by glutathione reductases (Mannervik, 1985). As indicated earlier, glutathione peroxidase is essentially inactivated by the decrease in activity of G6PD, which also affects the total storage of reduced glutathione (Figure 5.2).

The increased GPx content in the hemolymph of IMD-treated bees suggests a likely defense mechanism against increased production of hydroperoxides, which in turn may reduce cell membrane damage. Blahova *et al.,* (2013) observed increased GPx activity due to pesticide exposure in tissues with higher concentrations of this enzyme. The different mechanisms of action of GPx in the midgut and hemolymph may be due to the different baseline concentrations of this important free radical scavenging enzyme in the two regions. Ascorbic acid supplementation was found to increase the activity of GPx in both the midgut and hemolymph of worker bees. This is indicative of the ability of ascorbic acid to increase GPx production in various tissues independent of baseline concentration. This clearly indicates that ascorbic acid intake can attenuate the toxic aspects of IMD in various tissues.

Glutathione Reductase (GSR) is a key enzyme that is involved in the redox metabolic cycle of GSH along with GPx. GPx catalyzes the oxidation of GSH (reduced form) to GSSG (oxidized form). GSSG thus formed is reduced by GSR, which utilizes NADPH as the reducing factor (Jefferies *et al*., 2003). A significant reduction in GSR levels was observed in the hemolymph and mid gut tissues of worker bees which were exposed to IMD alone. Since the concentration of GSR in cells is much lower than that of GPx, any variation in the redox balance due to enhanced oxidative stress can cause a significant reduction in this enzyme levels. Based on our observations, we can suggest that GSR also follows the same scenario as

that of GPx (Figure 5.2). The increase in the activities of GPx and GSR observed in the VIT C supplemented group points to a higher level of antioxidant defenses to reduce the level of ROS.

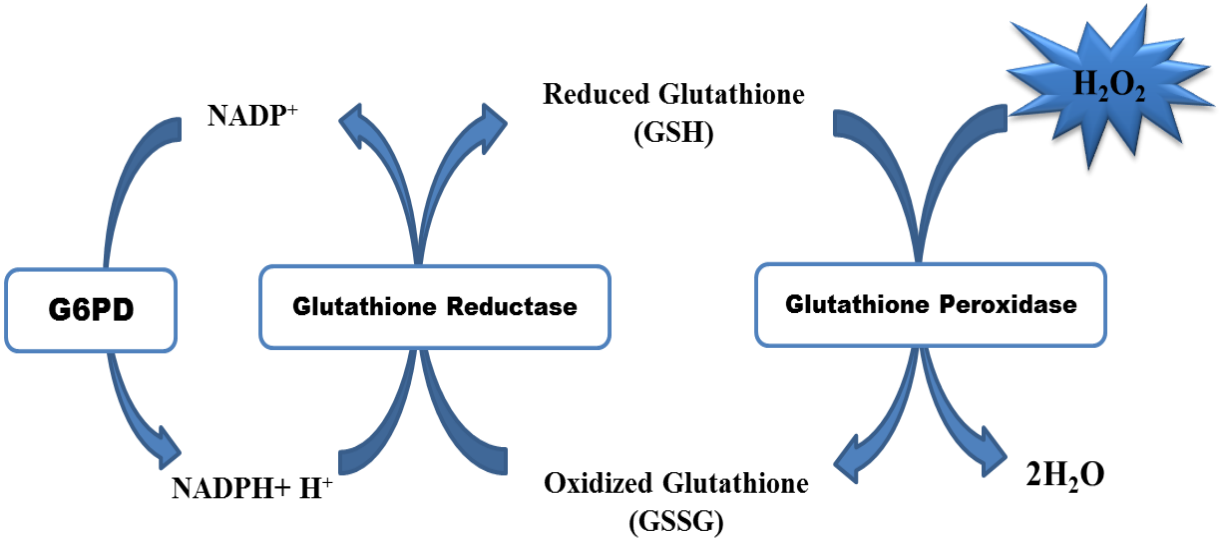


Figure 5.2. Schematic overview of the redox pathways involved in the removal of hydrogen peroxide. Glutathione peroxidase inactivates hydrogen peroxide at the expense of GSH, which is oxidized to GSSG. The enzyme glutathione reductase (GR) recycles GSSG to GSH using NADPH, and glucose-6-phosphate dehydrogenase (G6PD) restores NADPH.

The honeybee genome has a lower number of genes encoding enzymes involved in the detoxification of xenobiotics (Claudianos *et al.,* 2006). A lower number of genes encoding antioxidant proteins in the honeybee genome indicate that its ability to defend itself against ROS appears to be severely limited compared to other insects (Corona and Robinson, 2006). Therefore, the supplementation of ascorbic acid will lead to a considerable increase in the antioxidant system, which will attenuate the oxidative stress caused by IMD. In insects, the glutathione-ascorbic acid redox cycle enables the scavenging of hydrogen peroxide produced by enzymatic reactions. Ascorbic acid reduces H_2O_2 to dehydroascorbic acid (DHA), which is recovered by dehydroascorbic acid reductase (DHAR), simultaneously oxidizing glutathione. Reduced glutathione (GSH) is then restored by the subsequent action of GSR (Summers and Felton, 1993; Krishnan *et al.,* 2009). An increase in H_2O_2 concentration leads to increased activity of ascorbate peroxidase in honeybees to detoxify its hazardous effect (Korayem *et al.,* 2012). Insects also possess a glutathione transferase with

peroxidative activity (GSTpx) that can catalyze the oxidation of ascorbic acid (Enayati *et al.,* 2005). The activation of these enzymes observed in the present study is probably due to the activation of the glutathione-ascorbic acid redox cycle to scavenge the excess ROS induced by the pesticide (Figure 5.3).

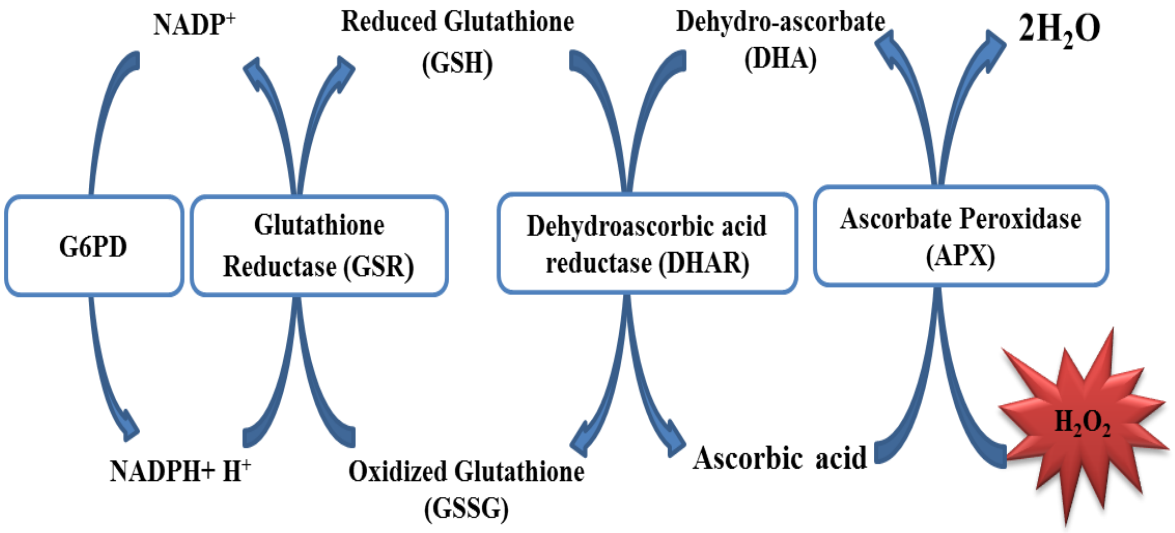


Figure 5.3. Schematic overview of the glutathione-ascorbic acid redox cycle. APX, ascorbate peroxidase; DHAR, Dehydroascorbic acid reductase; GSR, glutathione reductase; GSH, Reduced glutathione; GSSG, Oxidised glutathione, G6PD, Glucose 6 Phosphate Dehydrogenase.

An efficient antioxidant system is necessary in insects because their high metabolic rates lead to a large amount of free radicals under physiological conditions. The results of the present study showed that the activities of antioxidant enzymes including catalase, superoxide dismutase, glutathione S-transferase, and glutathione peroxidase in the hemolymph and midgut tissues of worker bees were significantly decreased on exposure to IMD as a single agent. However, their activities (CAT, SOD, GST, GPx and GSR) showed a significant increase in the VIT C supplemented groups. It is suggested that the activation of antioxidant enzymes is a defence mechanism to scavenge the excess ROS and mitigate the adverse effects caused by the pesticide.

5.4. Effect on the nicotinic acetylcholine receptors

Cholinergic synaptic transmissions are mediated in the insect nervous system by nicotinic acetylcholine receptors (nAChR) (Sattelle *et al.,* 2005). These receptors are targets for neonicotinoids, with imidacloprid exceptionally showing greater affinity for nicotinic acetylcholine receptors (nAChR) in the brain of honey bees (Nauen *et al.,* 2001). The binding of IMD to nAChR leads to changes in the normal signaling mechanism, resulting in overstimulation and inhibiting the action of acetylcholine (Matsuda *et al.,* 2001). A large number of nAChRs are distributed in the brain, and the receptors consist of five major subunits and many combinations of smaller subunits (Thany *et al.,* 2007). We focused on the expression of two toxicologically relevant nAChR subunits (nAChRα1 and nAChRα2) to investigate the effect of ascorbic acid supplementation on IMD- induced genotoxicity.

Our study showed the potential of imidacloprid in up regulating the expression of nicotinic-Acetylcholine receptors in the brain of worker bees. The nAChRs with binding sites for IMD are localized in the synaptic knob and cell body in the neurons of insects (Buckingham *et al.,* 1997). The nAChRα1 and nAChRα2 subunits are primarily associated with the transmission of cholinergic impulses. Some aminoacid residues in the α and β subunits of nAChRs contribute to the selectivity of neonicotinoids (Liu *et al.,* 2008). Because these receptors are associated with receptor-regulated signaling, binding of IMD could lead to upregulation of their expression, which may be a potential factor for neurotoxicity. Up-regulation of the expression of nAChRs due to IMD exposure may lead to a loss of function of these receptors, which in turn has undesirable effects on basic motor functions (Williamson *et al.,* 2014). In the brain of honeybees, oral exposure to imidacloprid for 72 h resulted in increased expression of *nAChRα1*. It is also reported that imidacloprid exposure induced expression of *nAChRα2* at 48 h, but in this case the effect subsided after 72 h of treatment (Christen *et al.,* 2016). Several studies conducted with different insects showed similar expression of nAChR upon exposure to neonicotinoids (Taillebois *et al.,* 2014, Markussen *et al.,* 2010, Wang *et al.,* 2015).

The results of the present study showed downregulation of nAChRα1 and nAChRα2 subunits in the ascorbic acid-supplemented group compared with the IMD-only exposed

group. IMD shows a higher degree of specificity for nAChRs in honeybee brain compared to the analogous receptors in mammals (Matsuda *et al.,* 2001) and therefore can perform a variety of activities at the homologous target site. IMD can act as competitors or partial competitors at the same target receptor site of nicotinic acetylcholine receptors (Brown *et al.,* 2006). Our results showed that ascorbic acid supplementation increased the expression of nAChR subunits, which could help to overcome the peroxidative damage to the membrane caused by the oxidative stress of imidacloprid. Dietary supplementation with ascorbic acid increased nAChR responses in rhesus monkeys by providing protection against cell damage in the brain region that is more susceptible to oxidative stress (Slotkin *et al.,* 2005). As shown by the observations in the present study, ascorbic acid supplementation can increase the expression of nAChRs in the brain, which protects brain tissue from the peroxidative damage caused by exposure to imidacloprid.

5.5. Effect on the ovary of queen bee

The queen is the only reproductive female in the colony. A healthy, high quality queen is essential for the survival of the colony (Amiri *et al.,* 2017). She is capable of laying a large number of fertilized and unfertilized eggs, and provides social stability for the colony through the pheromones she secretes. Therefore, the reproductive success of the queen bee is critical to the development and vitality of honey bee colonies (Winston 1987). Reports have shown that neonicotinoid insecticides negatively affect queen reproduction and physiology, which may result in lower queen success (Williams *et al.,* 2015). In our study, we observed that imidacloprid affects ovaries at molecular level, which may ultimately lead to reproductive failure (Figure 5.4).

5.5.1. Genotoxicity in queen bee

Cytochromes P450 are a large family of enzymes found in all life forms. By catalyzing oxidation processes, this enzyme system plays a crucial role in the metabolism of xenobiotics such as drugs, insecticides, and plant toxins (Feyereisen, 2012). AccCYP336A1 plays an important role in oxidative stress resistance in honeybees (Zhu *et al.,* 2016). Considering the significant role in antioxidant processes, we investigated the expression of AccCYP336A1 in the ovary. Upon exposure to imidacloprid, the transcripts of *CYP336A1*

showed lower expression in the ovary, indicating the genotoxicity caused by imidacloprid. It also shows that the ability of honeybees to respond to oxidative stress is impaired under these conditions. Previous studies have shown that imidacloprid at low concentrations downregulates the expression of genes related to antioxidation, immunity, and queen development. Queens exposed to low concentrations of imidacloprid exhibited reduced expression levels of P450 subfamily genes, suggesting that queens do not form effective antioxidants and defenses in response to synthetic pesticides (Chaimanee *et al.,* 2016). *CYP336A1* expression was downregulated in response to oxidative stress generated by heat and cold treatment (Zhu *et al.,* 2016).

Vitellogenin, the yolk precursor of insects, is a reproductive protein of honeybees but also plays a role in social functions. It is a multifunctional gene that plays an important role in hormone signaling, transition from nursing bees to foragers, stress resistance, and lifespan of honey bees (Amdam *et al.,* 2003). Vitellogenin can be used as a suitable biomarker for evaluating the sublethal effects of pesticides on honey bees (Abbo *et al.,* 2017). Our results showed that imidacloprid had negative effects on the vitellogenin gene in the queen ovary. This may lead to a reduction in lifespan, reproductive capacity, and increased oxidative stress. Since vitellogenin is a female-specific protein involved in the regulation of lifespan, the observed down-regulation of the vitellogenin gene may decrease the lifespan of the queen bee. This is confirmed by the observations of Nelson *et al.,* (2007). Vitellogenin is found in the fat bodies of queens and workers, as well as in functional ovaries (Cardoso-junior *et al.,* 2021). Fat bodies release vitellogenin protein into the hemolymph, which is then taken up by other tissues via the vitellogenin receptor protein (Li and Zhang 2017; Dohanik *et al.,* 2018). Therefore, the decreased expression of the vitellogenin gene in the ovary may adversely affect the reproductive potential of the queen bee.

The current results suggest that imidacloprid may have a negative effect on the reproductive function of queen bees due to increased oxidative stress in the ovaries. The decreased expression of *vitellogenin* and *CYP336A1* suggests that imidacloprid may affect the ovary of the queen through oxidative stress, revealing an imbalance between the generation of reactive oxygen species and the ability of the biological system to rapidly detoxify the reactive intermediates. Because detoxification is an energy-intensive metabolic

process, sublethal exposure to imidacloprid may cause increased energy consumption in honeybees (Abbo *et al.,* 2017). Reactive oxygen species formation may affect the histology and function of reproductive structures (Hoshi *et al.,* 2014).

The present study is the first experimental observation on the effect of ascorbic acid on the expression of *vitellogenin* and *CYP336A1* in *A. cerana indica* exposed to imidacloprid. Our results showed that both *CYP336A1* and *vitellogenin* were upregulated when queen bees of the ascorbic acid supplemented group were exposed to imidacloprid compared with the group exposed to IMD alone. These results suggest that ascorbic acid supplementation has a positive effect on the production of *vitellogenin* and *CYP336A1*, which protect the organism from oxidative damage.

5.5.2. Histological alterations in the ovary

The ovaries of the queen bee consist of ovarioles, which are morphologically divided into four areas: Terminal filament, germarium, vitellarium, and ovariole (Snodgrass, 1985). The vitellarium in the ovary of the queen from the control group consists of oocyte and trophocyte connected by a trophic stalk. The trophocytes have a large polymorphic nucleus and rich granular cytoplasm, as described by Snodgrass (1985) and Kozii *et al.*, (2022). The trophic stalk allows the transport of nutrients, RNA, and ribonucleoproteins from trophocytes to the oocyte during maturation (Berger and da Cruz-Landim, 2009; Cruz-Landim and Patrıcio, 2010; Snodgrass, 1985; Tanaka *et al.,* 2017; Kozii *et al.,* 2022). Trophocytes undergo programmed cell death after delivering their cytoplasmic contents to the developing oocyte (Tanaka *et al.,* 2017; Patrıcio and Cruz-Landim, 2008). As they move posteriorly along the ovariole, they steadily increase in size and eventually degenerate (Snodgrass, 1985).

The ovaries of queen bees exposed to IMD showed abnormal histology, such as perinuclear vacuolization and pyknotic nuclei in trophocytes, indicating early degeneration of trophocytes. The vitellarium also exhibited cytoplasmic vacuolization followed by oocyte degradation. The oocyte shrinks in the germarium, and intercellular gaps form in the cuboidal epithelium. Moreira *et al.*, (2022) also observed histological changes in the ovaries of *A. mellifera* queens, and the main abnormalities detected in the ovaries of these reproductive

bees treated with imidacloprid were degeneration and resorption of the ovariole contents, which probably affected fertilization and colony growth.

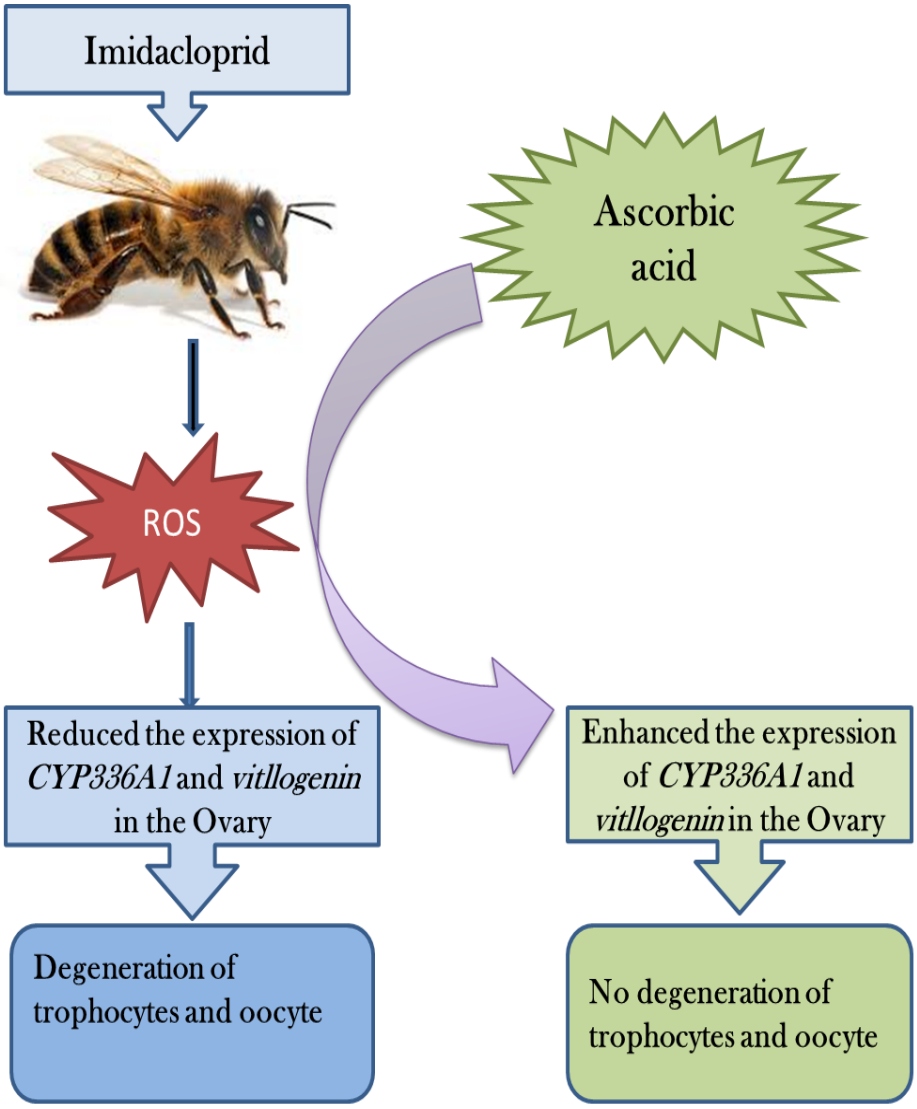


Figure 5.4. Schematic overview of the effect of supplementation of ascorbic acid on the ovary of queen bees on exposure to imidacloprid.

Histological studies have shown that ascorbic acid supplementation can help to reduce imidacloprid-induced toxicity in the ovary of queen bees. Histoarchitecture of the ovary of the ascorbic acid-supplemented group showed perinuclear vacuolization and pyknotic nuclei in trophocytes after exposure to IMD. In contrast, complete degradation of

the mature oocyte was not observed. Both the germarium and vitellarium showed perinuclear vacuolization. The cuboidal epithelium was not damaged in the germarium, and no intercellular gaps were not formed, as was present in the IMD-exposed group. According to Volkoff *et al.,* (2018), low dietary vitamin C leads to poor reproductive performance in females, as evidenced by fewer eggs, lower hatchability, and an increase in the number of malformed larvae in fish. The antioxidant function of vitamin C helps protect gamete cells by suppressing molecular oxidation, a reaction that can release free radicals that can degrade membranes and membrane integrity in both sperm and eggs of fish.

Apoptosis or autophagy or programmed cell death, is a critical mechanism for organ and tissue development as well as tissue homeostasis and immunity. Pesticide-induced oxidative stress can lead to programmed cell death in honeybee tissues (Kumar *et al.,* 2022). In the current study, the ovaries treated with imidacloprid showed degeneration with disorganized follicles and wrinkled ovary sheaths, while no complete deterioration of mature oocytes was observed in the ascorbic acid treated groups. During virus infection, queen bees showed ovariole degradation and autolysis. According to Liu, (1992) and Gauthier *et al.,* (2011), the accumulation of virus particles in the ovaries of queen bees or the accumulation of chemical toxins from large amounts of food consumed by the queen during her life, as well as the presence of numerous chemical residues in pollen above a certain threshold, can lead to a pathological condition in queen bees that, in extreme cases, can result in complete damage to the ovaries.

5.6. Effect on the hypopharyngeal glands

The decline in pollinator populations has caused widespread concern and prompted a number of studies to determine why bee colonies are disappearing (VanEngelsdorp *et al.,* 2009). One of the main causes is the indiscriminate use of pesticides, which have subtle deleterious effects on honey bee physiology and behavior. Imidacloprid has a number of negative effects on the ability of honey bees to perform functions such as learning, movement, and feeding (Decourtye *et al.,* 2004). In the present study, the toxicity of imidacloprid and the supplementation of ascorbic acid on the histological alterations in the hypopharyngeal gland of worker bees were investigated. The hypopharyngeal glands are important for the formation of royal jelly and the production of enzymes that metabolize

nutrients released during pollen digestion (Huang and Otis, 1989). Royal jelly is the main food for honey bee larvae in the early stages of development and throughout the lifespan of the queen. As it is provided by young worker bees, a decline in glandular activity affecting royal jelly production affects colony expansion, brood care, colony maintenance, and overall colony survival (Zaluski *et al.,* 2017).

Histologic examinations included evaluation and recording of the shape of the acini, the distribution of secretory vesicles, and the presence of extracellular space. Histology of the hypopharyngeal gland of worker bees revealed round-shaped acini with an irregular nucleus surrounded by eight to ten secretory vesicles. These observations are consistent with the findings of Suwannapong *et al.,* (2010) and Zaluski *et al.,* (2017).

Hypopharyngeal glands are susceptible to various stressors such as starvation, seasonal changes, and *varroa* infestation, which can lead to a decrease in activity and degeneration of the glands (Ali *et al.,* 2019; Yousef *et al.,* 2014). Exposure to imidacloprid resulted in changes in the cytological architecture of the hypopharyngeal gland of worker bees, such as malformations in shape and cytoplasmic vacuolization in the acini. This indicates the degenerative effect of imidacloprid in the early developmental stages of honey bees. These data confirm previous studies by Faita *et al.*, (2018) and De Castro *et al.,* (2020).

Gregorc *et al.,* (2018) reported the influence of imidacloprid treatment on HPG size and also on the expression of cell death. According to their observations, imidacloprid triggered extensive necrosis and an increased degree of programmed cell death. According to Heylen *et al.,* (2011), the hypopharyngeal gland showed decreasing size, granular texture, and disorganized cytoplasm, indicating early disintegration in life and the onset of foraging in older bees exposed to imidacloprid. In our studies, we observed vacuolization and lysis of secretory vesicles in the hypopharyngeal gland upon exposure to imidacloprid. These adverse effects on secretory cells may interfere with the normal activity of the gland and the associated defective production and secretion of royal jelly. This may contribute to the adverse effects of imidacloprid exposure on colony survival and normal functioning. According to Wessler *et al.,* (2016), long-term exposure to neonicotinoids also confirmed vacuolization in secretory cells and loss of secretory buds, resulting in a reduction of acetylcholine content in larval food.

Supplemental feeding supports brood development in honey bee colonies (Brodschneider and Crailsheim, 2010). Poor nutrition alone or in combination with other stressors can weaken bee colonies. Pollen supplements and vitamins promote hypopharyngeal gland development and brood number in bees (Zahra and Talal, 2008). Studies by Herbert, (1992) have shown that vitamin C deficiency can cause underdevelopment of the hypopharyngeal gland, which leads to an inadequate composition of royal jelly production by the nurse bees.

In the present study, histological examination of the hypopharyngeal gland of worker bees supplemented with ascorbic acid showed large and round acini with clusters of multiple secretory vesicles compared with the control group (10-12). Deseyn and Billen, (2005) reported that the activity and structure of the hypopharyngeal gland depend on age and specific tasks. They show a secretory cycle within the cells and secretion varies within the acini. Suwannapong *et al*., (2010) reported that the secretory activity of the hypopharyngeal gland peaks in nurse bees that have multiple secretory vesicles. Our observation showed that the number of secretory vesicles increased when supplemented with ascorbic acid compared to the control group. This suggests that ascorbic acid supplementation enhances secretory activity by increasing the number of vesicles. This is evidenced by the formation of clusters of secretory units in the hypopharyngeal gland in the experimental group. These observations confirmed that the early decay of hypopharyngeal glands was prevented by the supplemented ascorbic acid.

Zaluski *et al.,* (2017) suggested that pesticide-induced impairment of hypopharyngeal glands in nurses may be due to decreased nutrient digestion and absorption in nurses, which contributes to defective gland development. Imidacloprid can cause DNA damage, apoptosis, protein oxidation, and lipid peroxidation in non-target organisms by inducing the production of reactive oxygen species (ROS) (Ge *et al.,* 2015). The deleterious effect on the hypopharyngeal gland may be due to increased ROS production.

5.7. Effect on the midgut

The intestine, which is responsible for digestion and absorption, is an important area for morphological and toxicity studies. Since the midgut is the site of nutrient absorption, it

provides an access route for pathogens and toxins. Any morphological changes caused by the insecticide in this region of the digestive system may affect other physiological and behavioural processes (Williamson *et al.,* 2014; Catae *et al.,* 2014). Adult honey bee tissues that exhibit extensive cell proliferation are observed exclusively in the midgut epithelium (Ward *et al.,* 2008). Pesticide exposure negatively affects morphological and physiological traits as well as the peritrophic membrane, epithelium, and musculature. These changes disrupt normal behaviour, including food gathering, which adversely affect the survival of the colony (Carvalho *et al.,* 2013; Roat *et al.,* 2013). In addition, it impairs other physiological processes such as altered oxygen availability, synapse formation, and neuronal degeneration, which could be a cause of poor memory and learning abilities (Catae *et al.,* 2018). In the present study, we examined the histological architecture of the midgut (ventriculus) of honey bee queens and workers. We assessed the effect of ascorbic acid supplementation on the morphological changes of the midgut epithelium and peritrophic membrane upon exposure to imidacloprid.

5.7.1. Histological analysis of the midgut of worker bees

The histological analysis of the midgut of honeybees in the control group showed a typical morphology of the epithelium with a thin gelatinous layer. At the base of the epithelium, the nuclei are surrounded by lighter areas (hallos) with vacuolated cytoplasm. The midgut epithelium is the site of contact with pathogens and xenobiotics and it is responsible for detoxification of ingested xenobiotics (Higes *et al.,* 2013). In the wide lumen, we found that pollen grains are surrounded by numerous layers of peritrophic membrane. The peritrophic membrane serves as a mechanical barrier in the lumen. It consists of two areas: the endoperitotrophic space, which is located inside in contact with food, and the ectoperitotrophic space, which is located on the outside and in contact with epithelial cells (Teixeira *et al.,* 2015). The ectoperitotrophic space covers the surface of the intestinal epithelium and is composed of chitin and peritrophins, which ensure that the cells free from friction with the solid food as they pass through the ventricle (Lehane, 1997).

Histoarchitecture of the midgut of worker bees fed ascorbic acid showed a well-defined epithelium with a thick gelatinous layer. The nuclei are covered with hallos and the cell cytoplasm is less vacuolated compared to the control group. Complete cytoplasmic

vacuolization was not observed. This would indicate cell deformations in the epithelial lining (Bielenin and Ibek, 1980). In our study, the midgut of honey bees supplemented with ascorbic acid showed the presence of several layers of peritrophic membranes. It is associated with the duration of food intake (Crailsheim, 1988) and improves the efficiency of digestion (Bolognesi *et al.,* 2008). The consumed food can remain longer in the intestine and be better utilised by the body. The presence of significant amounts of peritrophic membranes has been demonstrated in pollen substitutes enriched with probiotics (Szymas and Przybyl 2007; Szymas *et al.,* 2012).

Due to the lack of chitin lining, the honey bee midgut is susceptible to harmful chemicals, infection, and malnutrition (Bielenin and Ibek, 1980; Maiolino *et al.,* 2014; Szymas and Przybyl, 2007; Szymas *et al.,* 2012). In our study, histological analysis of midgut epithelia exposed to imidacloprid revealed deformed epithelial tissue and completely ruptured peritrophic membrane. Isolated cellular aggregates were observed in the epithelial tissue along with defective and ruptured plasma membranes, resulting in vacuolization due to autophagy and cell necrosis. Similar observations were also made in previous studies. Bees orally exposed to neonicotinoid pesticides showed cytoplasmic vacuolization and a decrease in the number of their regenerative cells (Tapparo *et al.,* 2012). Pervez *et al.*, (2021) reported that there were significant morphological changes in the epithelial tissue of the midgut of honey bees treated with imidacloprid. This may adversely affect life expectancy, feeding and flight activity, colony cooperation, and learning. This may eventually lead to colony decline and extinction (El-Hassani *et al.,* 2008; Neumann and Carreck, 2010).

Microscopic examination of midgut cells from honey bee workers exposed to IMD shows degenerated epithelial cells and a completely ruptured peritrophic membrane, indicating tissue membrane damage. The damage could be due to a disturbed redox balance in the tissues caused by the increased production of free radicals due to the administration of IMD as a single agent. Diniz *et al.,* (2020) and Pizzaia *et al.*, (2020) found similar effects such as destruction of the peritrophic membrane and disorganization of the epithelium after oral administration of neonicotinoids to bees.

Histological analysis of midgut cells from ascorbic acid-treated worker bees exposed to imidacloprid revealed a thick epithelium and a multilayered peritrophic membrane. This

indicates the ability of ascorbic acid supplementation to protect tissues from IMD-induced toxic effects by properly maintaining cellular redox balance. The peritrophic membrane plays an important role in protecting epithelial tissue from the adverse effects of chemical and microbial components in the diet (Terra, 1988). Multiple layers of the peritrophic membrane in bees are associated with better utilization of nutrients (Szymas *et al.,* 2012, Crailsheim, 1988). Any changes in midgut epithelial cells and PM can be considered as indicators of environmental stressors (Pawert *et al.,* 1996). Therefore, maintaining a balance of the antioxidant system facilitates normal tissue function and survival.

5.7.2. Histological analysis of the midgut of queen bees

Histological analysis of the midgut of queen bees from the control group showed epithelium with less conspicuous epithelial folds. The peritrophic membrane appeared as stratified amorphous material. These features showed similarities with midgut histology described by Ferreira and da Cruz Landim, (2003) and Kovalskyi *et al.,* (2021) for fertile queen bees of the species *A. mellifera*. Since the type of food ingested by the bees has a significant influence on the structure of the midgut, the tissue of the queen was more developed than that of worker bees (Almehmadi *et al.,* 2010). Histological analysis of the midgut of queen bees from the ascorbic acid-fed group showed a well-structured, thick epithelium and an increase in the number of epithelial folds. This structural feature is due to the need to increase the surface area. The peritrophic membrane was not found as a layered structure, but as a thick layer of amorphous material.

Histological analysis of the midgut of queen bees showed adverse effects on the epithelium and peritrophic membrane when exposed to imidacloprid. The midgut of queen bees showed changes in the external musculature and cellular changes. The peritrophic membrane was ruptured and scattered throughout the lumen. Degenerated epithelial cells with ruptured basement membrane were found. These changes could lead to epithelial failure to recover and consequent failure to absorb nutrients. Moreira *et al.,* (2022) also reported the adverse effects on midgut epithelial cells of queen bees exposed to commercial imidacloprid.

In our study, the adverse effects of imidacloprid on ascorbic acid supplemented queen bees were less severe. The peritrophic membrane and epithelial cells were not completely degenerated, but little perinuclear vacuolization and the presences of large vacuoles were

observed. This suggests the ability of ascorbic acid supplementation to protect tissues from IMD-induced toxic effects by properly maintaining cellular redox balance. We also observed that when exposed to imidacloprid, ascorbic acid-supplemented queen bees showed intense proliferation of enterocytes in their midgut epithelium.

The observations from histological analysis correlate well with the results obtained from the antioxidant status of the tissues of worker bees. IMD was found to have toxic effects on tissues as a single agent by increasing oxidative stress in cells. The increased oxidative stress in cells caused by free radicals, damages cell membranes by the abstraction of electrons from membrane structures, which affects cell morphology. When compared with the activities of antioxidants, we can confirm that the redox status in worker bees was significantly affected by the administration of IMD as a single agent. This is an indication of the probable damage to midgut cell membranes by the increased production of hydroperoxides and other free radicals, since the antioxidant enzymes cannot neutralize them. On the other hand, it was found that ascorbic acid supplementation effectively increased the activities of antioxidant enzymes in the hemolymph and midgut tissues of worker bees, suggesting that worker bees from groups receiving ascorbic acid were more effective in counteracting the effects of oxidative stress.

The supplementation of ascorbic acid may contribute to the maintenance of redox balance in cells, which is the key factor in maintaining tissue morphology, as shown by the histological architecture of the tissues studied. Ascorbic acid is a general antioxidant in tissues and has been associated with a variety of metabolic activities in insects. Although there are specific enzymatic mechanisms for the removal of the reactive products, ascorbic acid content in tissues may be sufficient for chemical scavenging of superoxide and hydrogen peroxide from metabolically active sites where the required enzymes are in low abundance (Kramer and Seib, 1982). The study showed that exposure to imidacloprid can lead to toxicity, as evidenced by alteration of antioxidant enzymes and inhibition of AChE, histological changes in tissues, and alteration of gene expression. The evidence presented in the study and the data from the literature suggest that the oxidative stress induced by imidacloprid in honeybees can be alleviated by the supplementation of ascorbic acid.

Chapter 6.
Conclusions

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In the present study, it was hypothesized that dietary supplementation with ascorbic acid (VIT C) could reduce peroxidative damage in worker bees of *Apis cerana indica* exposed to sublethal concentrations of imidacloprid (IMD). Our results demonstrated the efficacy of imidacloprid in affecting redox balance in queens and worker bees. The resulting redox imbalance led to the accumulation of free radicals in the tissue compartments, which in turn resulted in the buildup of oxidative stress. The removal of electrons from cell membranes by the free radicals resulted in altered histological architecture and altered expression of genes.

The ascorbic acid supplementation as an adjuvant regulated the antioxidant defense system in tissues, as shown by the activities of scavenger enzymes and analysis of gene expression. The maintenance of normal tissue morphology by ascorbic acid is clear visual evidence of this. We conclude that ascorbic acid can be used as an effective natural supplement to alleviate the toxic effects of oxidative imbalance caused by pesticides.The following are the main findings of the study.

- Treatment with imidacloprid resulted in biochemical changes in the hemolymph of worker bees, as evidenced by an increase in total soluble protein content, a decrease in total carbohydrate content, and a decrease in the activities of glucose-6-phosphate dehydrogenase and acetylcholinesterase. Ascorbic acid supplementation restored AChE and G6PD activities to normal levels against the toxic effects of imidacloprid.
- Dietary supplementation of ascorbic acid has the potential to maintain the redox status in the midgut tissues and hemolymph of worker bees, as evidenced by the activation of antioxidant enzymes, a defense mechanism that scavenges excess ROS to mitigate the adverse effects caused by the pesticide.
- The present study demonstrated the effect of imidacloprid on nicotinic acetylcholine receptor expression in the brain of worker bees, as evidenced by the upregulation of *nAChRα1* and *nAChRα2* receptor subunit expressio**n**, but ascorbic acid supplementation maintained nicotinic acetylcholine receptor expression at its normal level.

- The decreased expression of *vitellogenin* and *CYP336A1* upon exposure to imidacloprid could affect the function of the queen ovary through oxidative stress, revealing an imbalance between the generation of reactive oxygen species (ROS) and the inability of the organism to detoxify the reactive intermediates. Ascorbic acid supplementation has the potential to maintain redox status in the ovary, as evidenced by up-regulation of *vitellogenin* and *CYP336A1*, which protect the organism from oxidative damage by imidacloprid.
- Changes in the histological architecture of the ovary and midgut of the queen bee indicate the effect of imidacloprid on reproduction and health of the queen bee, which negatively affects the survival of the colony. The addition of ascorbic acid may provide protection against the peroxidative damage induced by the sublethal concentration of imidacloprid, as evidenced by the maintenance of normal morphology of the tissues studied.
- The histological changes induced by imidacloprid in the tissues of the midgut and hypopharyngeal gland of worker bees reflect the effects on nutrient uptake and hypopharyngeal gland function, which in turn disrupt brood development and colony functioning.

Our results suggest that dietary supplementation with ascorbic acid has the potential to maintain redox status and thus may provide protective potential against peroxidative damage induced by sublethal concentrations of imidacloprid. In this regard, ascorbic acid as a food additive in the diet of *Apis cerana indica* could be an alternative to minimize pesticide-induced intoxication in hives, and it is important to continue research in this area to find alternatives for the benefit of beekeepers. Detailed studies in this area are needed to know the exact mechanisms behind the protective effects of ascorbic acid against the toxicity induced by neonicotinoids.

Chapter 7.
Recommendations

Chapter 7. Recommendations

Honey bees provide valuable ecosystem services and are considered the most economically important group of pollinators. Many studies have demonstrated the neonicotinoids as a major contributing factor for the decline of honey bee population, still substantial knowledge gaps remain on the sub lethal effects of neonicotinoids on foraging, reproduction and behaviors of adult bees. Based on the results of the study, we would like to propose the following recommendations.

- Currently approved uses of neonicotinoid insecticides should be re-evaluated to understand and reduce the risk to pollinators, especially honey bees. A more comprehensive assessment process may be implemented to address risks to honey bees and other pollinators, despite the key role these insects play in maintaining biodiversity
- Integrated pest management strategies can be applied in agricultural fields to reduce exposure and impact of insecticides on non-target species.
- Based on our study, ascorbic acid supplementation reduced peroxidative damage induced by imidacloprid. Advanced and detailed studies should be conducted to evaluate the effect of ascorbic acid supplementation on larval and brood development when exposed to imidacloprid.
- Studies are needed to evaluate the sublethal effects of imidacloprid and the effect of ascorbic acid supplementation on foraging and behavior of bees.
- The health of queen bee is critical to the proper functioning of the colony. Therefore, detailed studies are needed to demonstrate the effect of ascorbic acid supplementation on reproduction, fertility, health, queen longevity, and queen behavior.
- Honey is the most valuable product of honey bee colony. Bee products such as beeswax, bee venom, and royal jelly are commercially important products with medicinal properties. Exposure to imidacloprid contaminated royal jelly may alter the genetic characteristics of the queen. Studies need to be extended to evaluate the toxicity of imidacloprid and the effect of ascorbic acid supplementation on honey yield and composition of bee products.

Chapter 8.
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Chapter 8. References

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List of Publications

PUBLICATIONS

I. Papers presented in peer reviewed International Journals

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2. Syama P. S., & Kumar C. V., S. (2022). Evidence of diet supplementation with vitamin C protecting honeybees from Imidacloprid induced peroxidative damage: a study with *Apis cerana indica*. *Sociobiology*, *69*(3), e7763. https://doi.org/10.13102/sociobiology.v69i3.7763

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III. Papers presented in Conferences

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2. Syama, P. S., & Sreeranjitkumar, C. V; Queen raising behaviour of worker honey bees in a queenless colony. 42nd Annual Conference and National Symposium on Animal Behaviour, Biodiversity and Human future jointly organized by the Department of Zoology, University of Calicut and Ethological Society of India (ESI), 04-06, December 2018.

3. Syama, P. S., & Sreeranjitkumar, C. V; Raising of multiple Queens- A new initiative in Apiculture. National Workshop on 'Living on the edge..Disasters, predictions and management' organized by P.G. and Research Department of Zoology on 18, 19, 20 November 2019.

4. Syama, P. S., & Sreeranjitkumar, C. V; Effect of supplementation of ascorbic acid on imidacloprid induced toxicity in honey bee *Apis cerana indica*. International conference on Recent advances in Biosciences and Technology (RABT-21) organized by Marian Centre for Advanced Research (MCAR), research division of St. Mary's College, Thrissur, Kerala on 7th December 2021.

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RESEARCH ARTICLE - BEES

Evidence of diet supplementation with vitamin C protecting honeybees from Imidacloprid induced peroxidative damage: a study with *Apis cerana indica*

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Abstract

Neonicotinoids are one of the major stresses contributing to the decline in the population of honeybees. Worker bees are prone to various stress factors during foraging and are susceptible to Imidacloprid due to the reduction in the number of genes encoding for the major enzyme families responsible for the detoxification of toxins. The present study worked on the hypothesis that the dietary supplementation of Ascorbic acid (VIT C) could reduce the peroxidative damage in the worker bees of Apis cerana indica exposed to sub-lethal concentration of imidacloprid (IMD). Furthermore, we also evaluated the role and efficacy of VIT C supplementation on the cytoarchitecture of midgut tissues on exposure to IMD. Colonies of honeybees were maintained by providing sugar syrup to the control group and sugar syrup supplemented with 0.2% VIT C for the experimental group for six months. Worker bees from both groups were randomly collected and exposed to 0.001 mg/mL IMD. To study the peroxidative damage, the activities of various enzymes were analyzed. The activities of antioxidant enzymes including Catalase, Superoxide Dismutase, Glutathione S Transferase, and Glutathione Peroxidase in the hemolymph and midgut tissues of worker bees were significantly decreased due to exposure to IMD as a single agent. However, their activities showed a significant elevation under diet supplementation with VIT C. Histological examination revealed midgut tissue damage and the rupture of peritrophic membrane among the workers exposed to IMD as compared with the control group. The damage to the midgut was alleviated and the peritrophic membrane was found to be intact in the worker bees supplemented with VIT C. Our results indicated that the dietary supplementation of VIT C has the potential to maintain the redox status and thereby can offer protective potential against the peroxidative damages induced by the sub-lethal concentration of IMD.

Introduction

Multiple interacting factors including exposure to pesticides, attack of parasites and pathogens and habitat loss have been suggested as chronic sub lethal stress associated with the decline in the population of honeybees (Bryden et al., 2013). A group of neurotoxic insecticides, the neonicotinoids, has been singled out from all the stress factors due to its wide use in crop protection (Godfray et al., 2014). Neonicotinoids are synthetic analogs of nicotine which mimics the action of acetylcholine, the main excitatory neurotransmitter in the brain of honeybees (Casida et al., 2013). Neonicotinoids, which comprise imidacloprid, acetamiprid, clothianidin, thiomethoxam, thiacloprid, dinotefuran and nitenpyram were extensively used in seed treatments, soil applications and as foliar sprays to control crop pests (Blacquiere et al., 2012). They target nicotinic- Acetylcholine receptors (**nAChR**) and disrupt the functioning of the central nervous system

by overstimulation (Matsuda et al., 2001). This group of pesticides may cause behavioral problems in honeybees such as defective or delayed communication, navigation, homing and foraging (Henry et al., 2012) and adversely affect their immunity (Di Prisco et al., 2013). As these are systemic insecticides, their traces are found in nectar and pollen, bee products like bee bread, honey and beeswax (Blacquiere et al., 2012).

Imidacloprid (IMD) is widely used against insect pests because of its diverse application methods and lower toxicity to non-target organisms (Medrzycki et al., 2003). IMD and its metabolites were detected in pollen, honey samples, and from different honeybee body parts such as hemolymph, midgut, thorax and rectum (Suchail et al., 2004). Sub lethal effects manifested in bees exposed to IMD include inhibition in associative learning (Decourtye et al., 2004), abnormal foraging behavior (Yang et al., 2008), reduction in mobility and loss of communicative ability (Medrzycki et al., 2003). Considering the effect on metabolism, IMD impairs brain metabolism (Decourtye et al., 2004) and reduces mitochondrial activity (Nicodemo et al., 2014).

The midgut of honeybees is highly sensitive to poisonous substances (Bielenin & Ibek, 1980) and malnutrition (Szymas & Przybyl, 2007) due to the absence of chitinous lining. The midgut lumen has a protective covering called peritrophic membrane (PM) that surrounds the food (Snodgrass, 2018). In the lumen, PM acts as a semipermeable membrane and protects the midgut epithelium from mechanical damage, attack of pathogens (Lehane et al., 1997) and from toxic substances (Bielenin & Ibek, 1980). Metabolic resistance is one of the principal mechanisms used by insects to escape the adverse effects of natural and synthetic toxins (Esther et al., 2015). Organisms are endowed with a wide variety of endogenous antioxidative enzymes that protect cells from peroxidative damage, such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GSR) and Glutathione S-transferase (GST). As the honeybees have a lower number of genes coding for antioxidant proteins in their genome, the ability of honeybees to defend against ROS is strongly limited as compared to other insects (Corona et al*.,* 2006). The antioxidant enzymes in honeybees are efficient in detoxification of ROS and SOD acts as the first line defense against oxygen free radicals (Corona et al., 2006). H_2O_2 is eliminated by the action of CAT (Aebi, 1984) and GPx (Mannervik, 1985). GST conjugates the toxic compounds to glutathione to facilitate their removal from the tissues (Hinton et al., 1995; Jakoby, 1985; Lee, 1991).

Vitamin C is an important water-soluble antioxidant which can neutralize ROS and reduce oxidative stress (Verma et al., 2007). It can work from both inside and outside the cells to combat free radical damage and may act as a source of electrons to free radicals such as hydroxyl and superoxide radicals in order to quench their reactivity (Bendich et al., 1990; Bindhumol et al., 2003). Earlier studies showed that the dietary supplementation of VIT C positively influences the antioxidant defense and in turn reduces the winter mortality rate (Farjan et al., 2012; Andi & Ahmadi, 2014). The present study was designed to examine the protective effect of dietary supplementation of Ascorbic acid on the antioxidant defense system and histological alterations in the midgut of honeybee workers under IMD exposure.

Materials and methods

1. Experimental Protocol

1.1. Field colonies used in the experiments

The honeybee colonies of *A. cerana indica* used in the experiment were maintained in a domestic garden (Palakkad District, latitude-10.89, longitude-76.4). Experimental colonies were provided with 0.2 % VIT C in 250 ml sugar syrup (1:1 sugar and water) while the control colonies were provided with 250 ml sugar syrup alone. They were fed once in a week, throughout the experiment over a period of six months. The colonies were periodically examined for the viability of the colony.

1.2. Laboratory Experiment

Worker bees were randomly collected from the brood of control colonies and were divided into two groups. Out of this one group was maintained as control by providing sugar syrup alone (**Control)** and the other group was administered with a sub lethal concentration of 0.001 mg/mL imidacloprid along with sugar syrup (**IMD**). Similarly, the vitamin C supplemented bees were also grouped into two. Out of this one group was maintained as control with vitamin C supplementation (**VIT C Control**) and the other group was administered with imidacloprid at a sub lethal concentration of 0.001 mg/mL **(VIT C + IMD**) and all the groups were left undisturbed for an hour after treatment (the sub lethal concentration of IMD was achieved by evaluating LD_{50} as per the guidelines mentioned in OECD, 1998).

2. Enzyme assays

Ten worker bees were randomly selected from each treatment, and they were anesthetized by keeping them at 4 ºC for five minutes. The hemolymph was collected using microcapillary tubes after incising it dorsally between the 5th and 6th abdominal segment by using a sterile needle. The midgut was dissected out according to the protocol described by Carreck et al. (2013). They were pooled and homogenized in saline solution, centrifuged at 10,000 rpm for 20 minutes at 4 ºC, then the supernatants were decanted and used for enzyme assays. The enzymatic activity of Catalase (Luck, 1974), Peroxidase (Reddy et al., 1995), Superoxide dismutase (Paoletti et al., 1986), Glutathione S-transferases (Habig et al., 1974), Glutathione peroxidase (Rotruck et al., 1973), Glutathione Reductase (David & Richard, 1983) of the hemolymph and the midgut tissues were determined. The experiment was repeated three times.

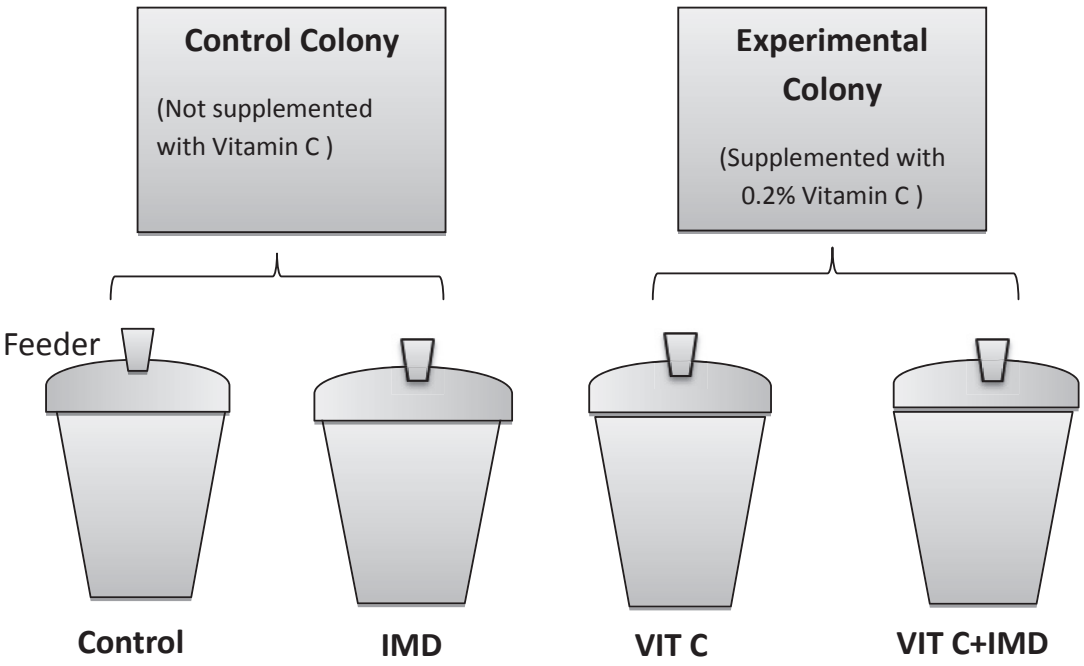

Fig 1. **Control**: Workers from control colony, **IMD**: Workers from Control colony exposed to **IMD** at 0.001mg/mL, **VIT C**: Workers from Experimental colony, **VIT C + IMD**: Workers from Experimental colony exposed to IMD at 0.001mg/mL.

3. Histological analysis

The midgut of worker bees from each treatment were dissected out and fixed in Bouin's fixative for the histological studies. Serial sections were dewaxed and dehydrated using different grades of alcohol and were double stained with Hematoxylin and Eosin. The histological analysis was done and photographs were taken by using a **LEICA DM 750** light microscope. Scaling and labelling were done by using Image J software.

4. Statistical analysis

A probit analysis was done for the LD_{50} calculation. Significant differences among treatments were identified by One Way ANOVA and pairwise analysis was carried out using DMRT (Duncan Multiple Range Test) at 5% ($p < 0.05$) level of significance with statistical software 'R' Version 4.1.1.

Results

1.Enzymes of Antioxidant System

1.1. Catalase activity

The exposure of honeybees to IMD significantly inhibited catalase activity in the hemolymph and midgut, since in this treatment the CAT activity was 4.7 ± 2.5 and 57.9 ± 13.1, respectively. There was a clear decrease in the CAT activity in comparison to the control (12.06 ± 3.38 and 101.1 ± 11.8, for hemolymph and midgut, respectively). When the diet was enriched only with VIT C, the CAT activity (34.9 ± 5.9 for hemolymph and 146.2 ± 0 for midgut) presented the highest and statistically significant effect. Finally, the supplementation of VIT C in the diet of honeybees exposed to IMD contributed to suppress the negative effects the insecticide, reestablishing the CAT activity to levels higher than in the control for the hemolymph and equal to the control for the midgut (Fig 2).

1.2. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was assessed in both hemolymph and midgut (Fig 3). No significant change ($p \geq 0.05$) was observed in the activity of SOD in the hemolymph of worker bees, when they were exposed to IMD (2.0 ± 0.005) compared to control (2.0 ± 0). In the midgut tissues, the IMD treatment resulted in a significant ($p \leq 0.05$) decrease of enzyme activity (2.01 ± 0.008). A significant increase in the SOD activity in the hemolymph and midgut of worker bees was noticed in VIT C supplemented treatment (2.06 ± 0.008 for hemolymph and 2.04 ± 0.02 for midgut) against the control (2.0 ± 0 for hemolymph and 2.03 ± 0.01 for midgut). When worker bees were exposed to IMD but the diet was supplemented with VIT C, the SOD activity was found to

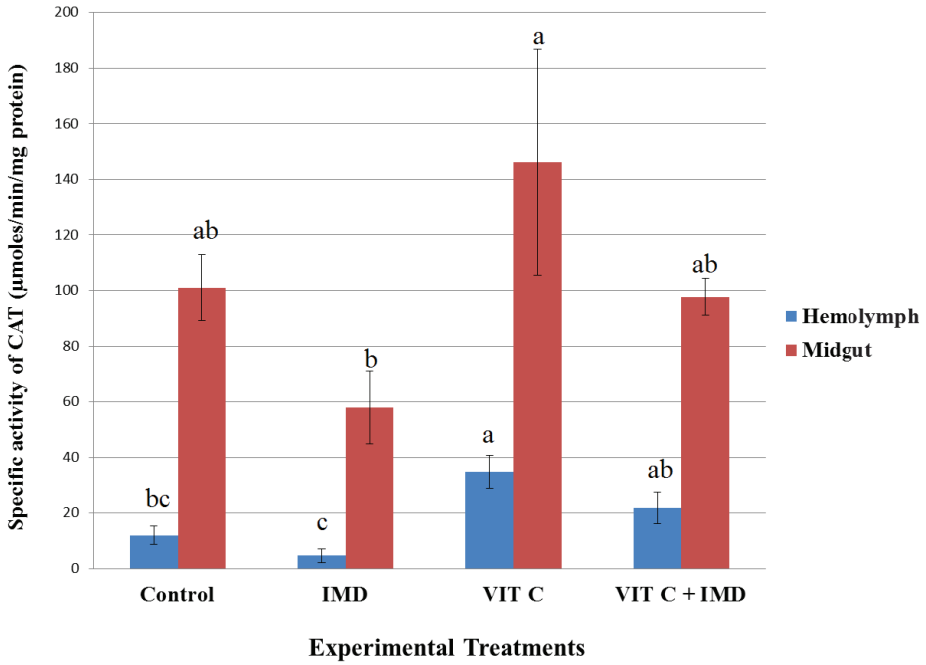

Fig 2. Specific activity of CAT in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

be increased significantly in the hemolymph (2.03 ± 0.01) when compared to control, but no significant difference was observed in the midgut in comparison to the control.

1.3. Peroxidase activity

Peroxidase (POD) activity was assessed both in the hemolymph and midgut tissues of worker bees (Fig 4). No significant difference ($p \geq 0.05$) was recorded in the activity of POD in the hemolymph of worker bees from treated groups and control. An increase of activity was recorded in IMD fed bees (2846 ± 1223), but no significant difference ($p \geq 0.05$) was found in other treatments. The IMD treatment resulted in a significant elevation ($p \leq 0.05$) of enzymatic activity in the midgut of worker bees (11660 ± 1725) as compared to the control treatment (2837 ± 18.02). A significant decrease of POD activity was observed in the midgut of worker bees from VIT C treatment (3055 ± 212.3) and VIT C + IMD treatment (5670 ± 515) when compared to IMD treatment (11660 ± 1725).

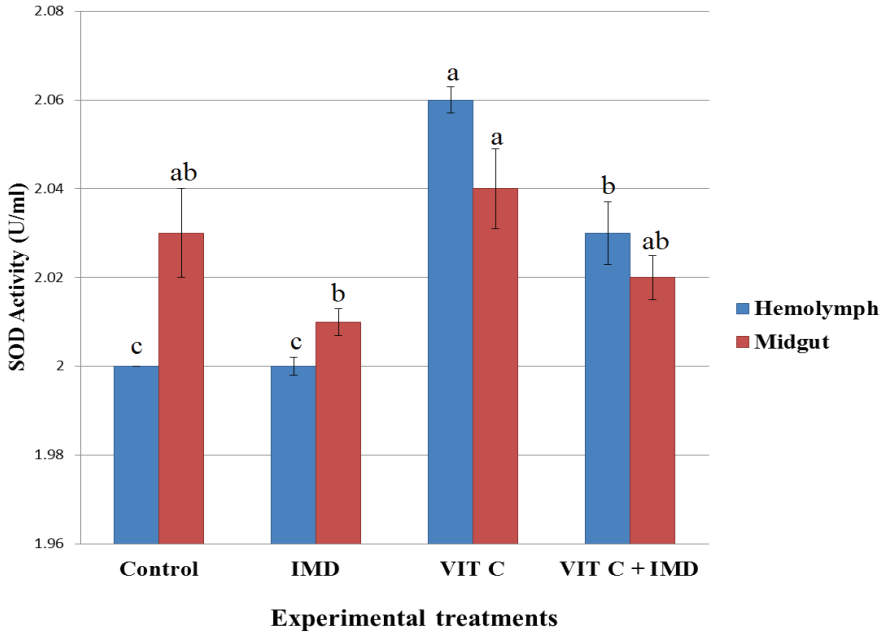

Fig 3. Specific activity of SOD in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

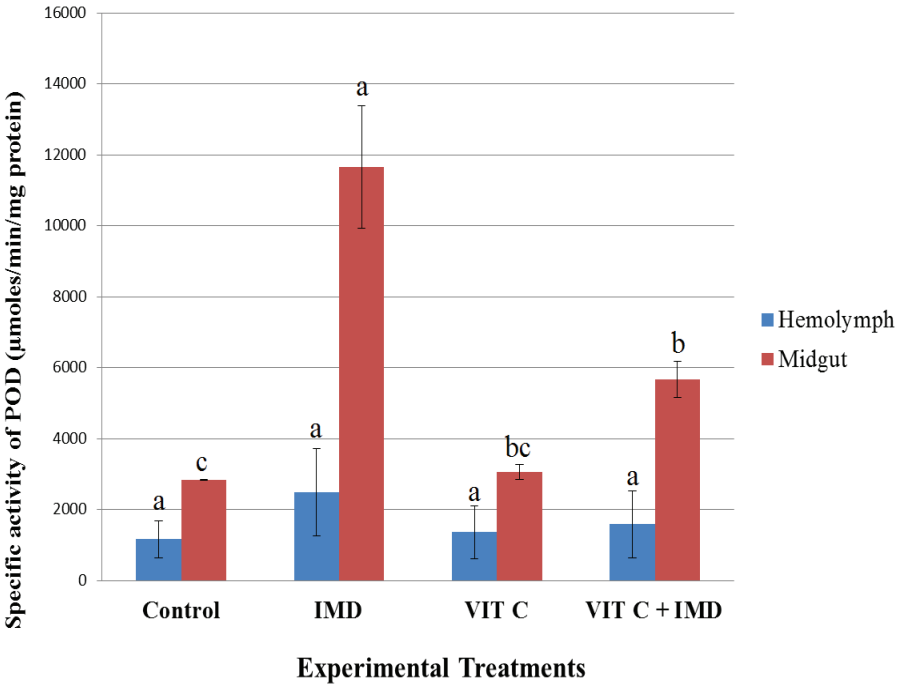

Fig 4. Specific activity of POD in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

1.4. Glutathione S-transferases activity

Statistical analysis performed on the data showed that worker bees in the IMD treatment had a significantly ($p \leq 0.05$) lower GST activity both in the hemolymph (2.24 ± 0.6) and midgut (0.7 ± 0.3) compared to the control (6.4 5± 1.0 and 1.01 ± 0.3). The worker bees from the VIT C treatment (11.4 ± 2.1 and 2.0 ± 0.3) and VIT C + IMD treatment (7.44 ± 0.9 and 2.6 ± 0.3) had a significantly ($p \leq 0.05$) higher GST activity both in hemolymph and midgut as compared with the control (Fig 5).

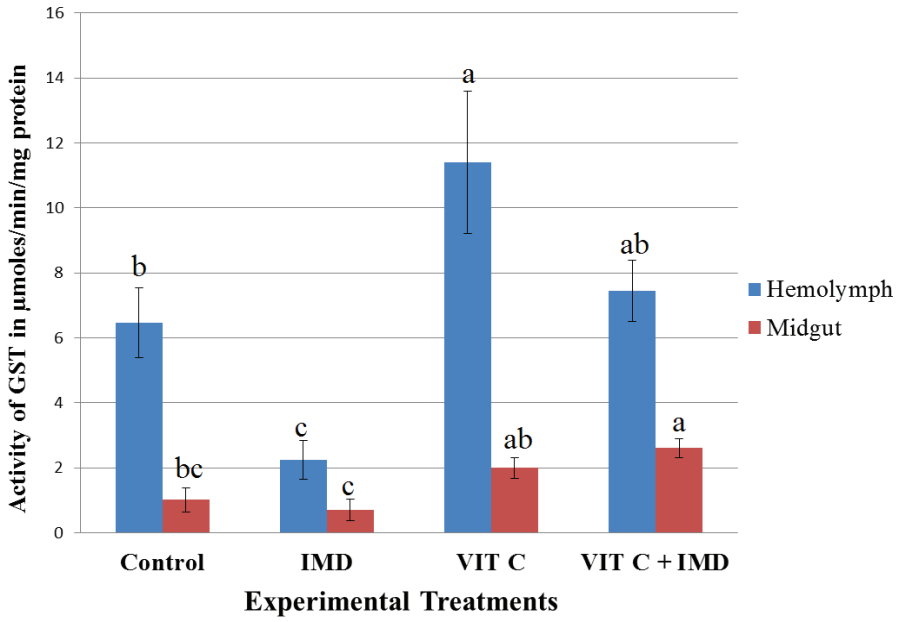

Fig 5. Specific activity of GST in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

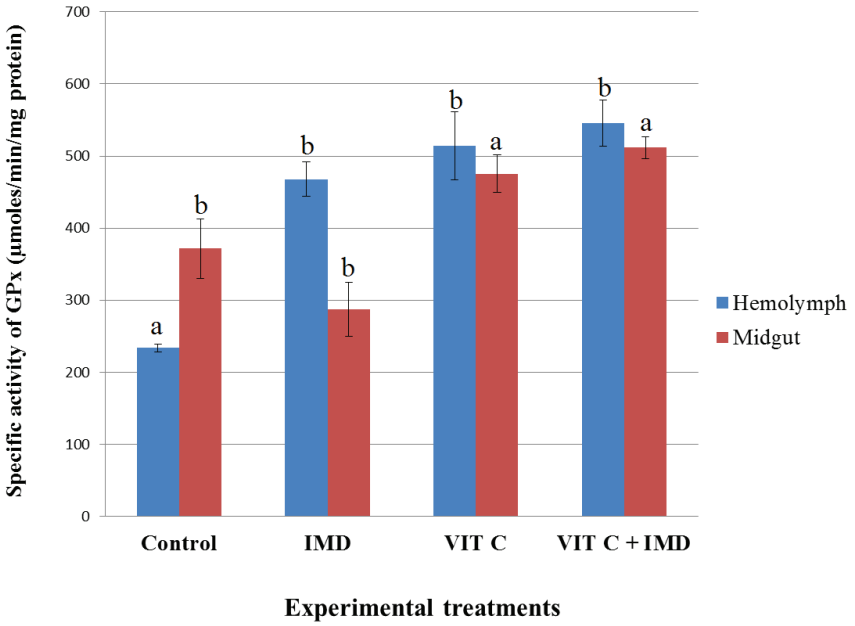

Fig 6. Specific activity of GPx in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

1.5. Glutathione peroxidase activity

The activity of the enzyme Glutathione peroxidase was measured in the hemolymph and midgut of worker bees (Fig 6). The activity of this enzyme in the hemolymph was significantly higher with the treatments with exposure to IMD and/or supplementation with VIT C, in comparison with the control. Regarding midgut tissues, the exposure of honeybees to IMD did not affect the enzyme activity. However, the supplementation of the diet with VIT C alone or combined with the exposure of honeybees to IMD significantly increased the enzyme activity.

1.6. Glutathione Reductase activity

GSR activity was measured in the hemolymph and midgut of worker bees (Fig 7). The statistical analysis performed on all data showed an effect of VIT C in increasing enzyme activity in the hemolymph (0.76 ± 0.09) of worker

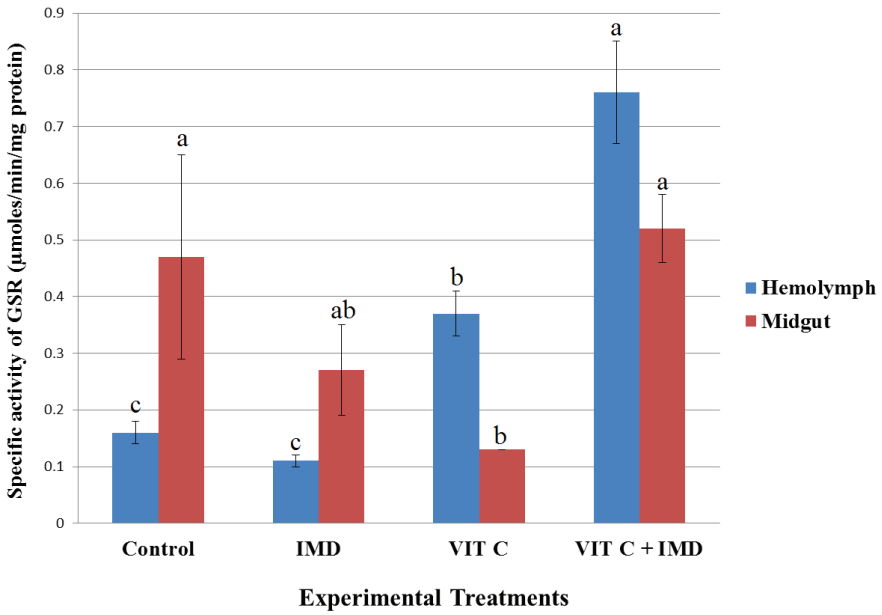

Fig 7. Specific activity of GSR in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

bees in comparison to the control (0.16 ± 0.02). The activity of the enzyme was found to be reduced when the worker bees were exposed to IMD.

2. Histological analysis

The histological analysis of the midgut of honeybees from the Control (Figure 8-A) showed a typical morphology of epithelium which contains a thin gelatinous layer, multiple layers of peritrophic membrane (PM) and a wide lumen between PM and epithelium. In some regions, the midgut epithelium was higher and, in some places, it was not well defined. At the base of the epithelium, the nuclei are surrounded with lighter areas (hallo's) with vacuolated cell cytoplasm. Pollen grains were surrounded by numerous layers of peritrophic membranes. Tearing of the PM from the gut epithelium was clearly visible in some regions. Intestinal lumen is wider in the control group.

In the Vitamin C supplemented group (Figure 8-B), the analysis of the midgut showed a well-defined epithelium with a thick gelatinous layer. The nuclei were covered with hallo's and the cell cytoplasm was less vacuolated. The epithelium was higher in some places and the epithelial folds were not well defined. Pollen grains present in the midgut region were covered with multiple layers of peritrophic membranes. Tearing of the peritrophic membrane was seen at some regions. Intestinal lumen was slightly wide.

Cyto-architectural analysis of the midgut of bees in the IMD treatment (Figure 8-C) showed adverse effects, where the peritrophic membrane was totally ruptured and the midgut contents were dispersed in the lumen. The gut epithelium was degenerated and without the gelatinous matrix, and the cells had large vacuoles.

The severity of the damage to the histological architecture was comparatively less on VIT C +IMD treatment (Figure 8-D). The thickness of the epithelial lining was found to be the same as in VIT C treatment, but large vacuoles were present in the epithelium. The peritrophic membrane was found intact, degeneration of epithelial cells and loss of gelatinous matrix were not observed.

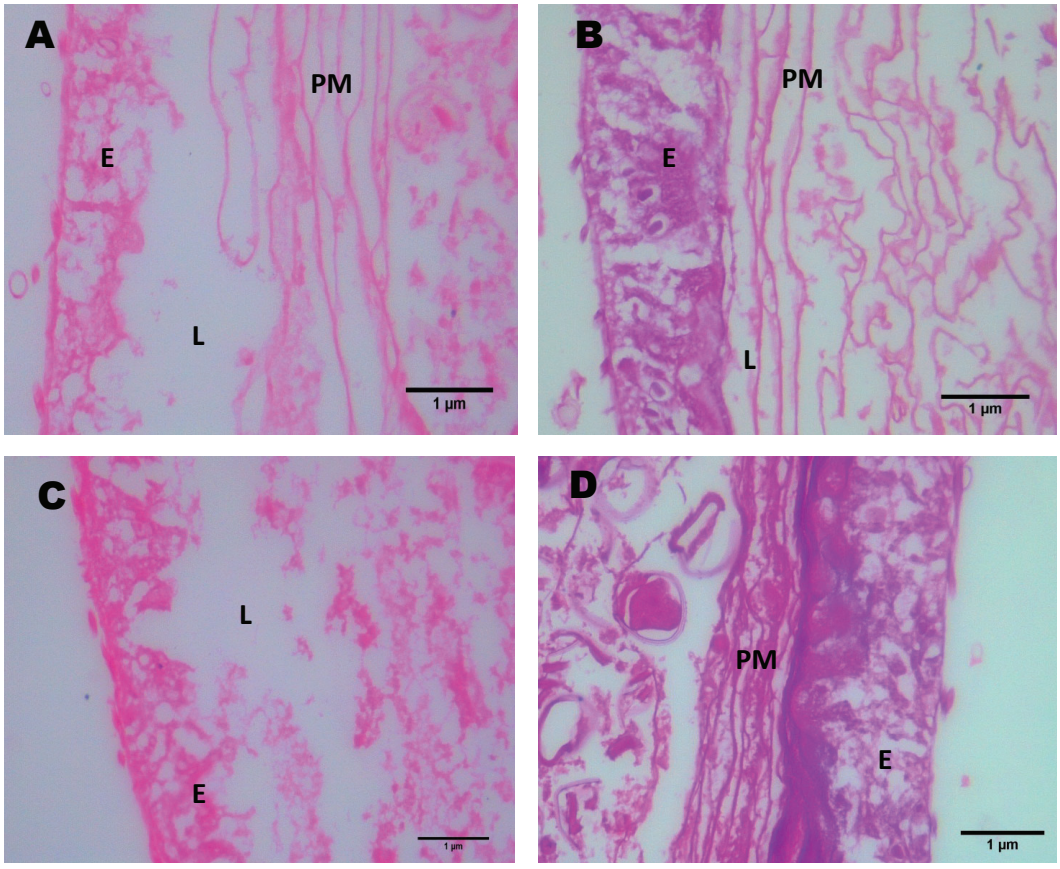

Fig 8. Histology of midgut cells of worker honeybee (***A. cerana indica***) stained with H & E. **A-D** includes, **A**. midgut cells of honeybee workers received sugar syrup presenting thin gelatinous layer of epithelium with wide lumen and intact peritrophic membrane. **B**. midgut cells of honeybee workers supplemented with Vitamin **C** showing well defined epithelium, slightly wide lumen and multiple layers of peritrophic membrane. **C**. midgut cells of honeybee workers exposed to **IMD** showing degenerated epithelial cells and completely ruptured peritrophic membrane. **D**. midgut cells of ascorbic acid supplemented worker bees exposed to imidacloprid presenting thick epithelium and multi layered peritrophic membrane. **E** Epithelium; **PM** Peritrophic Membrane; **L** Lumen.

Discussion

Oxidative stress refers to an imbalance in the redox status of the body due to the overproduction of free radicals beyond the capability of the antioxidant defense system to neutralize it. The uncontrolled scenario of free radical production may pose a serious threat to the survival of the body tissues due to the damage of the vital cellular components such as the membranes, lipids, nucleic acids, and proteins (Hodgson & Smart, 2001). Aerobic organisms are endowed with protective mechanisms comprising enzymatic and non-enzymatic antioxidants that are habitually efficient in blocking the detrimental effects of free radicals, including ROS.

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the key components to maintain the redox balance since they scavenge the excess of free radicals and can be the first line of defense against various types of such oxidative radicals. SOD and CAT are the enzymes which act as subsequent constituents in the antioxidant response system of the cells. CAT converts superoxide ($O2^-$) into H_2O_2 and then SOD converts the so formed H_2O_2 into H_2O and O_2. Various enzymatic free radical scavengers including catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), peroxidase, and superoxide dismutase (SOD) are reported to be present in vast quantities in bees (Weirich et al., 2002; Strachecka et al., 2014; Strachecka et al., 2017). The elevation in the levels of antioxidant enzymes may therefore be an important indication of an organism's endeavor to counter an induced oxidative stress.

The present study brings forth new insights to the capability of dietary supplementation of Vitamin C in diminishing the peroxidative damages generated by IMD. The specific levels of antioxidant enzymes in the honeybee *A. cerana indica* were studied to understand the tolerance and detoxification strategies against IMD toxicity. The present observations indicate that the exposure of honeybees to IMD had a significant effect on the activities of antioxidant enzymes. The activation of antioxidant enzymes observed in the present study is supposed to be a defensive mechanism to scavenge the excess ROS to alleviate the adverse effects induced by the pesticide.

The primary function of catalase enzymes is the conversion of hydrogen peroxide (H_2O_2) into H_2 and O_2. H_2O_2 is one of the most stable reactive oxygen species and plays a key role in the pathologies of numerous diseases, including Alzheimer's disease. Catalase is an antioxidant enzyme found in many cell types and is constituted by several sulf-hydryl groups (mainly of the amino acid, cysteine) in its internal structure. Neutralization of free radicals by this antioxidant is through the expenditure of its sulf-hydryl groups (Deisseroth & Dounce, 1970; Chance et al., 1979; Egaas et al., 1999; Milton, 2004; Enayati et al., 2005; Kirkman & Gaetani, 2007). In the present investigation we observed a significant reduction in the activity of catalase enzyme in both the midgut and hemolymph regions of worker bees exposed to IMD.

Down regulation of catalase activity is coupled with augmented defenselessness to oxidative stress. Uncontrolled free radical production may be due to the saturation of the available sulf-hydryl groups of catalase enzyme. This imbalance in the redox balance may in turn attack the catalase enzyme and can cause denaturation of its structure. The overall result is the damage to the cell membranes by H_2O_2 induced oxidative damages (Goth et al., 2004; Ho et al., 2004). Vitamin C as an adjuvant was found to elevate the levels of catalase enzyme significantly in both the midgut and hemolymph regions of worker bees. The protective potential of this vitamin is supposed to be due to its capability to reduce the free radical induced damages by either reducing the levels of free radicals or by stimulating the activity of catalase enzyme.

Superoxide dismutases (SODs) refer to a group of metalloenzymes that are present in all kingdoms of life. SODs serve as the front-line defense mechanism against tissue injuries due to reactive oxygen species (ROS). These proteins catalyze the dismutation of superoxide anion free radical (O_2) into molecular oxygen and hydrogen peroxide (H_2O_2) and thereby decreasing O_2 levels which otherwise can damage the cells at excessive concentrations (Younus, 2018). Our study showed a reduction in the activity of SOD in the hemolymph and midgut tissues of honeybees exposed to IMD. The reduced activity of the scavenging enzymes observed in the study reflects the excess of ROS generation due to the exposure to IMD. Our findings are in line with Kapoor et al., (2010). On the other hand, Vitamin C supplementation was found to enhance the activity of SOD in the corresponding group in the present study. The elevated level of SOD is supposed to be due to the boosting of the dismutation process by IMD which in turn can facilitate the elimination of ROS.

The major purpose of the peroxidase enzyme is the breakdown of H_2O_2 to nontoxic components (Thangudu & Su, 2021). A significant elevation in POD levels was observed in honeybees exclusively exposed to IMD. This elevation in peroxidase levels is an indication of the enhanced synthesis of peroxidase enzyme to counter the increased volume of free radicals in the tissues. Vitamin C as an adjuvant was found to cause a significant reduction in peroxidase enzyme levels and this probably indicates the capability of this vitamin in alleviating the stress generated by oxidative stress.

Glutathione peroxidase refers to an enzyme family endowed with peroxidase activity which is assigned with the biological role of protecting the organisms from oxidative damage. GPx defends free radical attacks at the expense of GSH. El-Gendy et al. (2010) reported that the main function of GPx is to reduce lipid hydroperoxides to their consequent alcohols and to reduce free hydrogen peroxide, thereby regulating the redox balance in the body. In the present study, we observed a significant reduction in GPx levels in the midgut of worker bees exposed to IMD. This is an indication of the probable damage of midgut cell membranes due to the enhanced production of hydroperoxides and other free radicals because of the failure of GPx in neutralizing them.

Our observations are in line with the findings of Kapoor et al. (2010) who also reported a reduction in GPx activity on IMD exposure.

The enhanced level of GPx in the hemolymph in IMD treated bees indicates the probable defense mechanism against the enhanced production of hydroperoxides, which in turn can reduce the damage of cell membranes. The different mechanisms of action of GPx in the midgut and the hemolymph may be due to the difference in basal concentrations of this key free radical scavenging enzyme in the two regions. Vitamin C supplementation was found to boost the activity of GPx in both the midgut and hemolymph in worker bees. This is an indication of the capability of Vitamin C in enhancing GPx production in various tissues, irrespective of the basal concentrations. This clearly indicates that the administration of Vitamin C in the diet of beehives subject to foraging in areas or agricultural landscapes with IMD application can alleviate the toxic aspects of this insecticide in various tissues. This line of investigation can be of great importance to the practice of apiculture and the prevention of colony decline or even collapse. Our study presents interesting results in support of diet supplementation as a strategy to counteract the deleterious effects of IMD in the metabolic defenses of honeybees against toxic agrochemical compounds.

Glutathione S-transferases (GSTs) comprise a family of Phase II detoxification enzymes that are assigned with the function of safeguarding the different cellular macromolecules from attack by reactive electrophiles. GSTs regulate the levels of an enormous range of electrophiles by conjugating them with glutathione (GSH). Glutathione conjugation is the primary step in the mercapturic acid pathway that facilitates the eradication of toxic compounds (Townsend & Tew, 2003). Observations from our study indicate the significant reduction of GST levels in the hemolymph and midgut tissues of worker bees exposed to IMD. This may be due to the enhanced utilization of GSH in neutralizing the larger volumes of electrophilic toxic compounds produced due to IMD administration. On the other hand, Vitamin C as an adjuvant was found to present a different scenario with a significant enhancement of GST levels in the respective groups. The enhancement of GST activity may be due to the alleviation of the levels of oxidative radicals by Vitamin C, which in turn resulted in the reduced usage of GSH. This elevated concentration of GSH can offer protection against cellular stress by scavenging the excess free radicals.

Glutathione Reductase (GSR) is a key enzyme that is involved in the redox metabolic cycle of GSH along with GPx, which in turn catalyzes the oxidation of GSH (reduced form) to GSSG (oxidized form). Once GSSG is formed it is reduced by GSR, which utilizes NADPH as the reducing factor (Jefferies et al., 2003). A significant reduction in GSR level was observed in the hemolymph and midgut tissues exposed to IMD alone. Since the concentration of GSR in cells is much lower than that of GPx, any variation in the redox balance due to enhanced oxidative stress can cause a significant reduction in these enzyme levels. It can be noted that Vitamin C supplementation was effective in enhancing the activities of these antioxidant enzymes in the hemolymph and midgut tissues. GSR also follows the same pattern of activity. Such a positive up regulation of antioxidant status along with the down regulation of oxidative stress may be the reason for maintaining the proper redox status in the hemolymph and midgut tissues.

The digestive system is the element of contact with pathogens and poisonous substances and is responsible for the detoxification of harmful substances (Higes et al., 2013). Histological analysis of the midgut epithelium may reveal the toxicity of ingested xenobiotics (Han et al., 2012). The midgut epithelial cells of bees fed with sugar syrup elicited normal appearance, the peritrophic membrane (PM) appeared visible and intact with normal thickness (Figure 8-A). The midgut cells of honeybees supplemented with Vitamin C elicited well defined epithelium, slightly wide lumen and multiple layers of the peritrophic membranes, indicating normal tissue morphology (Figure 8-B). Microscopic examination of the midgut cells exposed to IMD reveals degenerated epithelial cells and completely ruptured peritrophic membrane, indicating damage to the tissue membranes. The damage may be due to the irregular redox balance in tissues due to the enhanced production of free radicals on exposure to IMD (Figure 8-C) (Balieira et al., 2018; Yucel & Kayis, 2019). Peritrophic membrane plays an important role in protecting epithelial tissues from the adverse effects of chemical and microbial components in food (Terra, 1988). Any alterations in the midgut epithelial cells and PM can be considered as an indicator of environmental stress (Pawert et al., 1996).

Histological observations of the midgut epithelium of Vitamin C treated bees exposed to IMD showed a multi-layered peritrophic membrane. This indicates the capability of Ascorbic acid supplementation in protecting the tissues from IMD induced toxic effects by the proper maintenance of the cellular redox balance (Figure 8-D). Multiple layers of peritrophic membrane in bees are associated with better utilization of nutrients (Szymas et al., 2012; Crailsheim, 1988). The modifications in the cyto-architecture of gut epithelium give supplementary evidence for the protective role of VIT C against IMD toxicity.

Conclusion

The present study revealed the potential of imidacloprid in adversely affecting the redox balance in the midgut tissues and hemolymph of honeybees. The redox imbalance thus generated has resulted in the accumulation of free radicals in the midgut tissues, which in turn resulted in the building up of oxidative stress. The scavenging of electrons from the cell membranes by the free radicals has resulted in the altered morphology of tissues as evidenced from the histological

analysis. On the contrary, Vitamin C supplementation as an adjuvant was found to properly regulate the antioxidant defense system in the tissues as evidenced from the activities of scavenging enzymes. The maintenance of normal morphology of the tissues by Vitamin C serves as strong visual evidence for such regulation. Hence, we suggest that Vitamin C can be used as an effective natural supplement in relieving the toxic aspects of pesticide induced oxidative stress.

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Authors' Contributions

SPS: Conceptualization, methodology, formal analysis, investigation, writing and editing.
SCV: Conceptualization, methodology, supervision, writing and editing.

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Multiple Queen-Rearing in Queenless Colonies of the Asian Honey Bee *Apis cerana*

Syama Praveen and Chalilputhenveettil V. Sreeranjitkumar

Introduction

Queen-rearing is one of major objectives of commercial beekeepers to maintain colonies (Morse, 1994). Worker bees are capable of raising a new queen mainly for colony reproduction or emergency queen-rearing to replace a failing or missing queen (Seeley, 1985). Sudden decline in queen mandibular pheromone availability induces supersedure (Fell & Morse, 1984; Pettis et al., 1995). Rearing of queen bees can be performed in queenright colonies and queenless colonies, however a higher effectiveness can be achieved in queenless colonies (Morse, 1994). Queen-rearing in queenright colonies utilizes frames of brood raised above a queen excluder in a strong colony and grafting 12-18 h old larvae into queen cell cups next to the brood in the upper chamber (Wilkinson & Brown, 2002). The factors that determine the rearing of queens are the age of the transferred larvae, the number of young worker bees available in the colony, availability of food, number of drones etc. In addition, the quality of the resulting queen is determined by various factors such as weather conditions, availability of nectar and pollen, and even grafting methods play key role in the rearing of good quality queens (Kaftanoglu & Kumova, 1992). During grafting, worker larvae are kept in artificial queen cups and placed in queenless colonies for acceptance. Doolittle (1889) developed a successful method of rearing queens in *Apis mellif*era colonies by grafting larvae. The queen cells with larvae were accepted, fed, and finished in queenright colonies without a queenless period. Laidlaw (1979) and Laidlaw and Page (1997) described the general principles of a queenright starter-finisher method for queen-rearing in *A. mellifera* colonies. This method is commonly used to commercially produce royal jelly or queens. The objective of the present study is to develop a suitable method of queen-rearing in queenless colonies of *Apis cerana* without grafting larvae.

Materials and methods

For this experiment we maintained *A. cerana* colonies in Palakkad District, Kerala (latitude 10.89, longitude 76.4) under natural conditions. Standard procedures of maintenance were adopted for all colonies and we ensured the presence of sufficient adult workers, drones, sealed worker, and drone cells. The colonies were periodically checked to prevent them from any infectious diseases. Four colonies having sufficient adult workers, drones, sealed worker cells, sealed drone cells, and eggs were selected and marked to conduct the present experiment for three months from October to December. They were subjected to colony division by introducing a new method (Figure 1). From the selected colony, the frame having the queen bee along with two other frames were selected and were placed in an empty bee box. Thereafter both boxes were filled with three empty frames to build new combs. The hive with the queen bee was then

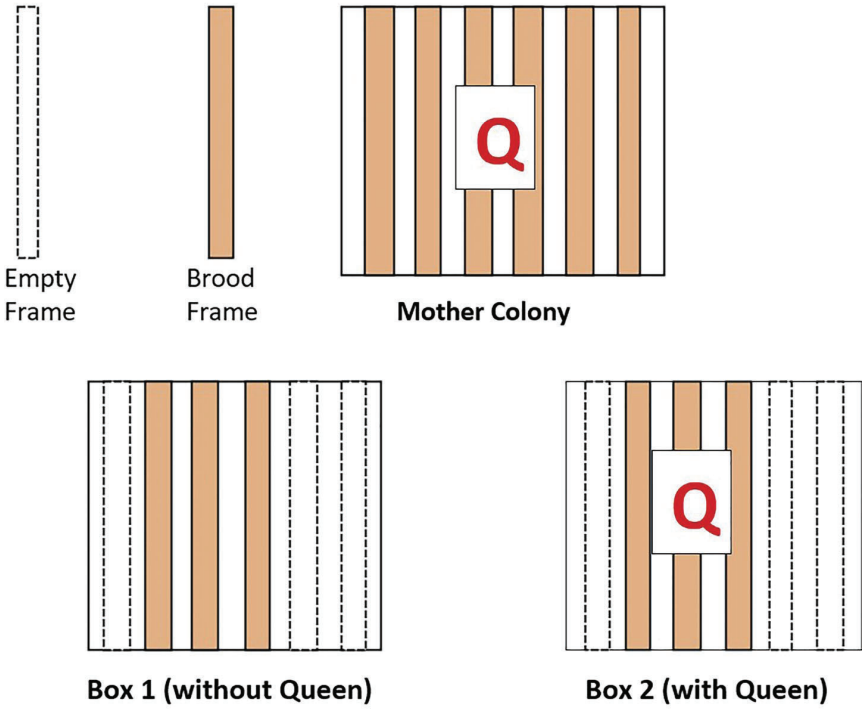

Figure 1. Representation of the division of the selected colony to make a colony without queen and a colony with queen.

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transferred to a new locality. All the colonies were fed with sugar syrup (1:1) throughout the experimental period. Periodical observations were made to ensure the progress of queen cell construction by worker bees in the queenless colonies. Mature queen cells (queen cells in which the queens are ready to emerge) were removed from the comb with care on the 9th day after division. Two ripe queen cells (uncaged) were left on the comb to study the emergence and acceptance of uncaged queen cells. Removed queen cells were placed in queen cups and fixed on a free frame (Figure 2). All queen cups were then capped with queen cages (Figure 3). The frames having the queen cages were re-introduced into the same queenless colonies (Figure 4). Caged and uncaged queen cells were periodically observed to assess the acceptance and to monitor the emergence of queens. We compared the emergence rates of caged and uncaged queens. An overview of the experimental procedures and observations is presented in Table 1.

Figure 2. Mature Queen cells were removed.

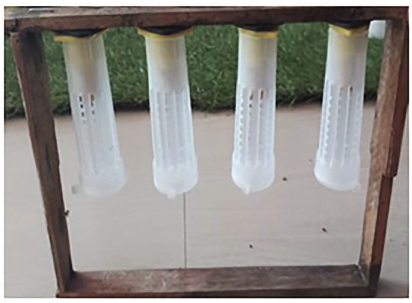

Figure 3. Mature queen cells capped with queen cages were placed in a separate frame and re-introduced to the same colony.

Figure 4. Frame with queen cages.

Table 1. Experimental procedures and observations.

DAY	EXPERIMENTAL PROCEDURES AND OBSERVATIONS
1	Colony division and preparation of queenless starters with three frames instead of six
2	Artificial feeding with 1:1 sugar syrup
3	Observed that the workers started to construct more than one queen cell in a colony.
5	Observed more than one queen cell in a colony.
7	Artificial feeding with 1:1 sugar syrup
8	Observed multiple ripe queen cells.
10	Mature queen cells were removed and placed in queen cages. Retained remaining ripe queen cells (uncaged) on the comb.
11	Emergence of queens from the uncaged queen cells.
13	Emergence of queens in the queen cages.
14	Emergence of queens in the queen cages.

Results

The emergence rates of uncaged queen cells and caged queen cells in queenless colonies are shown in Table 2. The average emergence rate of queen bees from uncaged and caged queen cells was found to be 50.00% (n = 8) and 56.25% (n = 16), respectively. In the present study, we found that queen bees emerged from the uncaged queen cells on the eleventh day (Figure 5). Queen bees in the caged queen cells emerged on the thirteenth and fourteenth days of observation (Figure 6 and 7). Both caged and uncaged queen bees emerged in the queenless colonies. Worker bees accepted the new uncaged queen in all queenless colonies under investigation. The queenright colonies obtained after colony division were functioning normally.

Discussion

Queen-rearing is one of the important requirements for successful beekeeping. As it is very common situation, that colonies lose their queen, beekeepers adopt different methods for rearing queens in queenright and queenless colonies. Little information is known on the queen-rearing of *A. cerana*. While many studies focused on queen-rearing in queenright colonies of *A. cerana*, few have focused on queen-rearing in queenless colonies. In previous reports on rearing of queen larvae in queenright colonies, the queen was excluded from the brood-rearing part of the hive (Laidlaw & Eckert, 1962). Lensky (1971) demonstrated that close contact of the queen with the grafted larvae had no inhibitory effect upon their acceptance and a physical barrier separating the queen from the larvae is not necessary. The conditions that determine the success of mass-rearing of *A. cerana* queens were studied by Abrol et al. (2005) using artificial queen cell cups for grafting larvae.

Wongsiri et al. (1990) practiced queen-rearing with *A. cerana*. According to them, ripe queen cells can be taken under natural conditions from colonies preparing to swarm. Ripe queen cells can be removed 10-11 days after cell building began and before a virgin queen emerges. An emerged virgin queen will destroy other queen cells and inhibit queen cell production. One queen cell (often the longest and most perfect) may be left in the colony to re-queen the colony. In the present study we found one queen per colony that emerged from the two uncaged queen cells of each colony, even in the presence of caged queens. Our method has the advantage that the queenless colonies can be used for queen-rearing while maintaining the old queen in the queenright colony. This can increase the productivity, honey production and the pollination services of bees by increasing the number of colonies. Another reason to perform this method is to prevent the colonies from swarming.

Commonly adopted methods of queen-rearing are based on grafting worker larvae, which are then transferred in natural or artificial queen cups using grafting tools. Dodologlu et al., (2004) compared some characteristics of queen honey bees reared using the Doolittle method from that of the natural method. The difference between the two methods regarding pre-oviposition period were not significant. In the present investigation, we grafted mature queen cells to queenless colonies instead of grafting larvae in order to reduce the risks in handling of larvae during grafting. The queen emergence rate in the present study was found to be 50%, which is similar to the findings recorded by Cengiz *et al.* (2009) under grafting method.

Conclusion

In the present study, we have report on a queen-rearing method for queenless *A. cerana* honey bees. This method can be

Table 2. Emergence rate of queen cells in queenless *A. cerana* colonies.

Queenless colony	Total number of queen cells	No. of queen cells destroyed	No. of Mature queen cells removed	No. of mature cells left (uncaged)	No. of mature queen cells grafted and caged	Emergence rate of queen cells (uncaged) (%)	Emergence rate of queen cells (caged) (%)
Colony A	8	2	4	2	4	50	50
Colony B	6	0	4	2	4	50	75
Colony C	10	4	4	2	4	50	50
Colony D	7	1	4	2	4	50	50

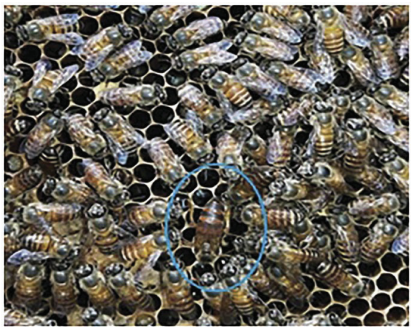

Figure 5. *A. cerana* queen emerged from an uncaged queen cell.

Figure 6. Emergence of a caged *A. cerana* queen on day 13.

Figure 7. Emergence of a caged *A. cerana* queen on day 14.

adopted for queen-rearing without any difficulties of grafting of larvae. We are introducing a new method for rearing multiple queens from a selected *A. cerana* colony for generating multiple queens required for research purposes and for conducting experiments. This method is suitable for producing multiple queens for re-queening a colony or providing queens for a queenless colony that will be helpful for beekeepers. Beekeepers can easily adopt this method to make splits and to increase colony numbers.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Effect of supplementation of Ascorbic acid on the comb building behaviour of worker bees (*Apis cerana)* during colony division

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Abstract

Honey bees are social insects which lead a fascinating colonial life. *Apis cerana* is an important bee in apiculture. They play an important role in maintaining food security and biodiversity through their pollination services. There is a steady decline in the population of *Apis cerana* due to many reasons. Climate change, use of pesticides, deforestation, pathogenic infections are the major threats for bee population. Their colony consists of a queen, thousands of workers and a few male drones. Worker honey bees, the slave females in the bee colony are always engaged in comb building, hive cleaning, pollen collection, nectar collection, honey making, nursing queen bee and larvae. In the present study we have studied the effect of supplementation of Ascorbic acid on the comb building behavior of *Apis cerana* during colony division. Honey bee colonies were constructed in suitable places and standard procedures were maintained for all colonies. Bee colony with full strength of adult bees was divided in to two colonies, one having Queen and one without queen. Queen right colonies after colony division were selected for further studies. Control colonies were provided with sugar syrup and experimental colonies were supplemented with Ascorbic acid sugar syrup. We observed that in the experimental colonies comb building was increased when compared to that of control group when supplemented with ascorbic acid. This behavior of worker bees can be utilized for increasing the brood area after colony division for the overall growth of the colony.

Keywords: Apiculture, comb building behaviuor, ascorbic acid, *Apis cerana.*

Introduction

Honey combs are mass of wax cells built by worker honey bees to care their larvae and to store honey and pollen. The wax is produced by eight wax-producing glands in the abdominal segments of worker bees. The wax is released from the abdomen as wax scales, which are used for comb construction. Honey comb in the lower chamber forms the brood which contains egg, larvae, pupae. Queen, the only reproductive female in the colony lays egg in each cell. Fertile queen lays thousands of eggs per day. Egg hatches to larvae, which is fed by the worker bees. Larva then enters in to pupal stage and then come out of the cell as adult. The brood is cared and monitored by nursing worker bees. Honey comb in the upper chamber of bee box forms the super, in which workers will store honey. Comb building is an important job of worker bees because they need comb to maintain their colony.

Honey bees require proteins, carbohydrates, lipids, vitamins and water. Bees collect nectar as their carbohydrate source and pollen as their protein source from flowers.During rainy season, beekeepers supplement sugar syrup for the bee colonies to compensate the inadequate supply of nectar from flowers. There is a need for supplemental food as the modern land use practices reduce dependable nectar and pollen supply [1].

Materials and methods

Apiary construction and management

Artificial bee hives were constructed in suitable places.Standard procedures were

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adopted for all colonies, which is periodically checked to exclude the presence of honey bee diseases. Study was conducted during the period of colony division, from October to December.

Colony selection

Bee colony with full strength of adult bees was selected for the study. Before selecting bee colonies, we have ensured the presence of sufficient number of adult workers, sealed worker cells, adult drones, sealed drone cells and eggs in the comb cells.

Colony division

Selected colonies were subjected to division. All colonies have six frames of comb in their brood chamber. During division of the selected colonies, queen bee along with three frames of comb is placed in another bee box. Now one colony is a queen right colony and one colony is a queen less colony. Then both the colonies are provided with three free frames. Queen right colonies after division are placed in a different location. Control and experimental queen right colonies were set in the same location. Control and experimental queen less colonies were set in another location.

Feeding

Control colonies are fed with 50% sugar syrup and experimental colonies are provided with 0.2% ascorbic acid supplemented sugar syrup. The sugar syrup was provided *ad libitum* to all the colonies every alternate day.

Results

We observed a busy schedule for workers for the construction of new comb in the free frames in all the colonies. We had observed that in the experimental group, workers were very active in building new comb as compared to that of control group. Table 1 shows the comb building behavior shown by different groups during feeding period. Experimental groups started comb building in the first week after division. But control groups started only in the second week. Experimental groups completed comb building in all the three frames by the fourth week. Control groups took five weeks to complete all the three frames. Table 2 shows the total brood area of control and experimental groups during the test period. Total comb area is found to be highest in the test colonies which are supplemented with Vitamin C(Figure 1). The total comb area of Queen Right test colonies is significantly high when compared to that of all other groups. Figure 2 shows the extra combs constructed by the worker bees in the Queen Right colony when supplemented with Vitamin C. These extra combs are found to be attached to the top of the bee box.

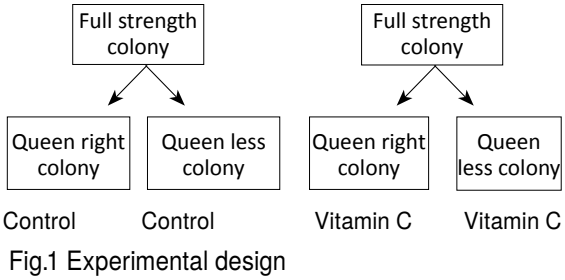

Fig.1 Experimental design

Fig. 2. Additional comb constructed by worker bees in Queen Right colony supplemented with Vitamin C

Discussion

In the present study we have studied the effect of supplementation of Ascorbic acid on the comb building behavior of *Apis cerana* during colony division. Table 1 shows the behavior of worker bees in control and experimental groups during the feeding period. During the first week after division we observed that in control groups, worker bees are focused on building platform in the first frame. But in experimental colonies, worker bees already constructed platform and started to extend new comb. In the Queen less colonies workers constructed queen cells to raise a new queen. Table 1

Table 1. Comb building behavior of control group and experimental group during feeding period

	Control (Queen right)	Control (Queen less)	Test colony (Queen right)	Test colony (Queen less)
Week 1	Started comb building in frame 1	Started comb building in frame1. Three queen cells present.	Started comb building in frame 1	Started comb building in frame 1. Presence of more than three queen cells.
Week 2	Building comb in frame 1	Building comb in frame 1.presence of new queen.	Completed comb building in frame 1.started comb building in frame 2	Building comb in frame 1 and started comb building in frame 2. Presence of new queen.
Week 3	Completed frame 1 and Started comb building in frame 2.	Completed frame 1 and started in frame 2.	Completed comb building in frame 2 and started comb building in frame 3.	Completed frame 1 and frame 2 .started comb building in frame 3.
Week 4	Completed frame 2 and Started comb building in frame 3.	Completed frame 2 and started frame 3.	Started building combs towards the top of the box.	Completed frame 3.
Week 5	Completed comb building in frame 3.	Completed frame 3.	Attached extra combs to the top of the bee box.	

Table 2. The total brood area of control and experimental groups during test period.

	Control (Queen right)	Control (Queen less)	Test colony (Queen right)	Test colony (Queen less)
Week 1	Frame 1-0 Frame 2-0 Frame 3- 0	Frame 1-0 Frame 2-0 Frame 3-0	Frame 1- 20cm^2 Frame 2- 0 Frame 3- 0	Frame 1- 12cm^2 Frame 2- Frame 3-
Week 2	Frame 1- 40cm^2 Frame 2-0 Frame 3-0	Frame 1- 24cm^2 Frame 2-0 Frame 3-0	Frame 1- 91cm^2 Frame 2- 60cm^2 Frame 3- 0	Frame 1- 40cm^2 Frame 2- 12cm^2 Frame 3- 0
Week 3	Frame 1- 187cm^2 Frame 2- 21cm^2 Frame 3-0	Frame 1- 150cm^2 Frame 2- 32cm^2 Frame 3-0	Frame 1-150cm^2 Frame 2- 126cm^2 Frame 3- 105cm^2	Frame 1- 132cm^2 Frame 2- 130cm^2 Frame 3- 36cm^2
Week 4	Frame 1- 187cm^2 Frame 2- 160cm^2 Frame 3- 44cm2	Frame 1- 192cm^2 Frame 2- 165cm^2 Frame 3- 18cm^2	Frame 1- 204cm^2 Frame 2- 176cm^2 Frame 3- 150cm^2	Frame 1- 165cm^2 Frame 2- 160cm^2 Frame 3- 140cm^2
Week 5	Frame 1- 187cm^2 Frame 2- 176cm^2 Frame 3- 180cm^2	Frame 1- 192cm^2 Frame 2- 187cm^2 Frame 3- 160cm^2	Frame 1- 204cm^2 Frame 2- 192cm^2 Frame 3- 150cm^2	Frame 1- 180cm^2 Frame 2- 187cm^2 Frame 3- 176cm^2

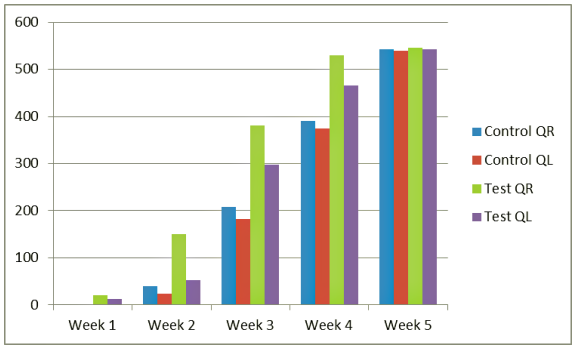

fig. 3. The total brood area of control and experimental groups during test period.

shows that when Queen less colonies are provided with Vitamin C, they contructed more number of queen cells when compared to that of control Queen less colonies.

Table 2 shows the total brood area of control and test groups during the feeding period. Significant increase in the total brood area was observed in test groups by week 4, which was supplemented with Ascorbic acid. Ahmadi Andi *et al*[2] studied the influence of vitamin C in sugar syrup on brood area, colony population, body weight, and protein in bees. They fed the control group with sugar syrup while experimental groups with different concentrations of soluble Vitamin C. They found that the highest average brood area was in the experimental group provided with 2000 mg/L Vitamin C. Herbert J [3] studied the effect of dietary vitamin C on brood rearing of honey bees using both free-flying and confined colonies. In this study they reported that colonies showed significantly more broodwhenthe diet was supplemented with 2000 mg/L ascorbic acid. Marek Farjan*et al*[4] reported the diet supplementation with vitamin C positively influenced some of the physiological and biochemical indicators in emerging worker bees. These results suggest that vitamin C can be recommended as a natural, safe, and relatively cheap diet supplement, elevating resistance to stress factors of wintering bees and spring generation of worker bees.

Conclusion

The present study indicates that supplementation of Vitamin C to the colonies after colony division shows a significant increase in the total brood area when compared to that of control colonies which are provided with sugar syrup.Total brood area increase in the bee colonies can be considered as an indicator of colony growth. So the supplementation of Ascorbic acid containing sugar syrup can be used to enhance the colony growth after colony division.

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