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ASSESSEMENT OF CHLOROPHYLL PHOTOTOXICITY ON HONEY BEE, APIS MELLIFERA (HYMENOPTERA: APIDAE).

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Abstract

This study evaluated the eco-toxicological parameters of two chlorophyll derivatives, copper chlorophyllin (Cu-chlorophyllin) and magnesium chlorophyllin (Mg-chlorophyllin) on viability of honey bee. Chlorophyll and its derivatives have a wide range of applications such as coloring in food additives and photopesticide against medical and agricultural pests. The results of Cu-chlorophyllin and Mg-chlorophyllin using different concentrations in honey bee's feeding bait indicated their efficiency on honey bee's viability as a function of concentration and exposure time. Honey bees' mortality rate was 10% within the range 10-6 -7x10-6 M/L and reached 25% at 10-5 M/L compared to 5% mortality in control samples. The LC50 of both derivatives showed the Mg-chlorophyllin was less toxic than Cu-chlorophyllin on bee's viability. The maximum mortality was 14% & 21% for Mg-chlorophyllin and Cu-chlorophyllin respectively. Semi-field experiment revealed that the natural activity of honey bee workers outside the hive reduced the effect of chlorophyll derivatives on honey bees' viability of laboratory testing obtained data.

Key words: Egyptian honey bee, Copper chlorophyllin, Magnesium chlorophyllin, Toxicity.

Introduction

Ecological and biological systems were in balance with each other but this natural balance is distorted through human activities by sharing and using products which kill beneficial and harmful organisms at the same time (Chauzat, 2009). The most famous new control methods are the biological control and the photodynamic by using photochemicals for control the population of different types of pests. These photosensitizing dyes exhibit several advantageous over chemical toxicants for pest control (Ben Amor and Jori, 2000). Chlorophyllin derivatives are successfully implemented as a tool to control the different species of mosquito larvae in a semi-field investigation (Abdel-Kader and El-Tayeb, 2012). Chlorophyll derivatives produce a reactive oxygen species (ROS) and its accumulation effect depending on the light exposure dose, and the exposure time. Chlorophyll derivatives have a wide range of applications as coloring in food additives (Viera *et al,* 2019) and photopesticides (Dokmak *et al,* 2021) against medical and agricultural pests (Abdel-Kader and El Tayeb, 2014). There were about 20,000 bees of 150 genera in seven families (Caron, 1990). *Apis mellifera* (family Apidae) originated in Eastern Africa and then spread abroad (Charles *et al,* 2006).

The accumulated chlorophyllin in insect tissue upon exposure to sunlight created reactive oxygen species (ROS) generated by energy transfer from excited triplet state of trisodium chlorophyllin to ground oxygen state within light exposed (Johnson and Carey, 2014). The insecticides cytotoxicity affected bees by photodynamic process and ROS stress re-action (Abdel-Kader and El-Tayeb, 2012). Thus, toxicity to honey bees from airborne insecticide dust during planting of insecticide-coated seeds (Marzaro *et al,* 2011) and increased pesticide residues in pollen and nectar from seed treatment with systemic neonicotinoids (Zhu *et al,* 2019).

The impact of pathogens, pesticides and environmental stressors weaken bees immunity and their physiology effect (Van Engelsdorp and Meixner, 2010). Chemicals that constituted pesticides can affect the honey bees immune health, their behavior, and even reduce or destroy their natural hormones (Chauzat *et al,* 2009).

Many chlorophyllins have powerful photodynamic sensitizers, characterized by a high quantum yield for the generation of singlet oxygen, a cytotoxic oxygen derivative which leads to oxidative stress for the insect cells and tissues (Uchoa *et al,* 2015). Oxidative stress theory showed that accumulation of reactive oxygen species elicits oxidative stress and physiological damage contributes to aging, health and lifespan of an organism, including insects (Williams *et al,* 2008). Honey bees underwent an oxidative stress due to their age-dependent foraging activity. The bees' wing muscles comprised *ca.* 35% of total body mass and contract at about 240 beats per second (Harrison *et al,* 1996). Its flight increased metabolic rates up to 100-folds as compared to resting behavior, and thus induced a great amount of ROS within mitochondria of the flight muscle cells, and within hive all were exposed to pro-oxidants through feeding and interactions with the external environment (Suarez *et al,* 2000). Alaux *et al.* (2011) reported that increased oxidative stress in bee queen and workers caused physiological changes that impact colony health and bee longevity. ROS are generated within the biological system to modulate the cellular activities such as cell survival, stressor responses, and inflammation, and thus bees, along with all aerobic organisms, must possess some sort of mechanism to counteract the effects of this oxidative stress. The innate capacity to mitigate effects of oxidative stress was achieved via antioxidant system and bee health was affected by minimizing oxidative stress.

The present study aimed to evaluate the effect of copper chlorophyllin, magnesium chlorophyllin on the Egyptian honey bee, *Apis Mellifera* L.

Materials and Methods

The present study was carried out between December 2015 and October 2019, in Department of Entomology, Faculty of Science, Cairo University and Department of Laser Application in Metrology, Photochemistry and Agriculture (LAMPA), National Institute of Laser Enhanced Sciences (NILES), Cairo University. Honey bee hive was established in Faculty of Agriculture, Cairo University.

Rearing of honey bee: Laboratory experiments were carried out with honey bee foragers of *Apis mellifera* L. The adult workers were obtained from Apiculture station, Faculty of Agriculture, Cairo University, Giza, Egypt, where honey bee colonies were maintained according to the standard commercial technique in the field. Foraging bees were considered the most ecologically relevant when they start performing external tasks (Picard-Nizou *et al,* 1995). Extensive literature confirms that foragers are those higher than 20 days of age in a typical colony of honey bees (Winston, 1987). Based on farming records, no obvious diseases were observed on units or colonies, and no hives were treated with any pesticides. This was confirmed during the collection of bees. Foraging workers were collected in special foam containers before treatment. Worker honey bees were collected from the front of the hives into a clean and large plastic bag. Then transferred into the containers and kept in good condition, and transported to the laboratory in 30 min. The bees were kept in experimental foam cages (10×7×12 cm) in groups of 50 at 25±2 °C with 65±5% RH, a photoperiod of 12:12 (Light: Dark), and fed on 50% (w/v) sucrose solution or chlorophyllin derivatives solution (Fig 1).

Cu-chlorophyllin and Mg-chlorophyllin stock solution: 1- Tri-sodium copper chlorophyllin a water-soluble sodium copper salt from chlorophyll by replacing magnesium atom at central ring with copper. 2- Tri-sodium magnesium chlorophyllin a yellow green powder, soluble in water giving transparent solution, slight soluble in alcohol and chloroform. Stock solution was prepared by dissolving 0.066gm of Cu-chlorophyllin or Mg-chlorophyllin in 9ml of distilled water & 1ml of NaOH (0.1 M). Solution was stirred overnight. Final solution was adjusted to PH = 7 and chlorophyllin derivatives solution was filtered and measured by absorption spectrophotometry.

Table 1: Chemical structure of sodium Copper Chlorophyllin and sodium magnesium Chlorophyllin

Sodium Copper Chlorophyllin		Sodium Magnesium Chlorophyllin	
Chemical Formula	C34H31CuN4Na3O6	Chemical Formula	C34H31MgN4Na3O6
Molecular Weight	724.15	Molecular Weight	684.91

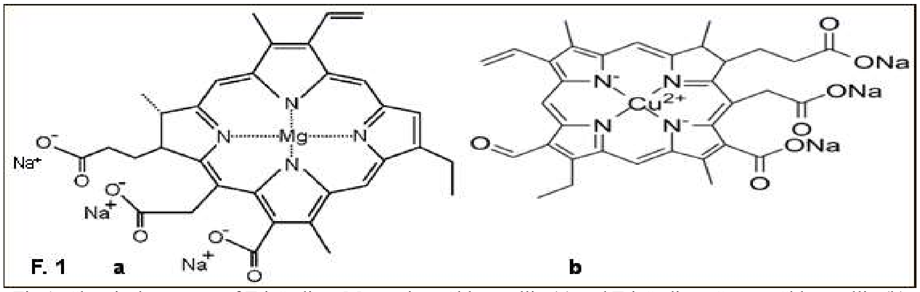

Fig.1: Chemical structure of Tri-Sodium Magnesium Chloropyllin (a) and Tri-Sodium Copper Chloropyllin (b)

Final stock solution of cu-chlorophyllin and mg-chlorophyllin were $0.9x10^{-2}$ M/L & $0.96x10^{-2}$ M/L respectively. Serial dilutions (10^{-3}, 10^{-4}, 10^{-5} & 10^{-6} M/L) of concentrations were prepared by taking aliquots of the solution diluted by distilled water according to M1xV1 = M2xV2. As M1: stock solution, M2: diluted solution, V1: volume of stock solution and V2: volume of diluted solution.

Toxicity study: For determination of LC_{50} of photosensitizer (cu-chlorophyllin or mg-chlorophyllin,), saturated sugar of chrolophyllin stock solution was used dilutions and offered to the worker bees for 24hrs in dark and exposed to direct sunlight, flounce rate measured by dosimeter average of intensities during exposure time. The mortality percentage was monitored at time intervals after light exposure for 1, 3, & 6hrs. The mortality was corrected by Abbott's formula (1925), and statistically computed (Finney, 1971).

Dark and light control experiments: In case of dark control experiments, the honey bee workers were incubated for feeding on a serial dilution of Cu- or Mg chlorophyllin in sugar solution for one week in viability experiments or 3 days in biochemical experiments. The dark experiment, were done to show the non-toxic effects of the dyes itself. the bees were treated by all concentrations of the chlorophyllin derivative and kept in dark during the experimental time. In case of light control experiments, groups of honey bee workers were exposed to all conditions of experiment except cholrophyllin incubition. The experiment was replicated three times with 20 bees for each replicate.

Effect of different concentrations on viability: In this experiment 36 cages of honey bee were used to test different concentrations (1e-6, 3e-6, 5e-6, 7e-6, 9e-6 & 1e-5 M/l) of tri-sodium magnesium chlorophyllin and tri-sodium copper chlorophyllin. Each cage was divided to 6 treatment cages (T1-T6) and 3 control cages. The C1: light control where honey bees were exposed to sunlight without treatment. The C2: honey bees were treated and were kept in dark all-over experiment. C3: honey bee was neither treated nor exposed to sunlight (normal control). All were kept in laboratory conditions under close observations.

Cages T1, T2, T3, T4, T5, T6 & C2 were treated for 24hrs feeding media, and were offered normal sugar solution and exposed to sunlight. After sunlight exposure the cages were transferred to the lab conditions and the honey bee viability were monitored for one week after. The light and normal control cages were feed on sugar solution all times.

Effect of different exposure time: 16 cages of bees were exposed to sunlight (1, 3, & 6 hrs) of tri-sodium magnesium chlorophyllin and copper chlorophyllin treated honey bee. Each treated cage was divided into (T1-T3) & 1 light control, exposed to sunlight without treatment, each of which were twice replicated. Cages T1, T2 & T3 were exposed to tri-sodium magnesium chlorophyllin or copper chlorophyllin) for 24hrs in food media, and then offered normal sugar solution and exposed to sunlight intervals of 1, 3, & 6hrs respectively. Then, they were transferred to lab and viability was tested after a week. Light control cages were feed on sugar solution, and exposed to sunlight for 6hrs.

Effect of different elapsed time of release: The 24 cages were elapsed for fixed concentration of tri-sodium magnesium chlorophyllin or copper chlorophyllin agents at different time to release bees' body. Each cage was divided to 5 cages (T1-T5) & 1 light control) and repeated in duplicates.

Bees cages were feed on tri-sodium magnesium chlorophyllin or copper chlorophyllin for 24hrs in food media, replaced with normal sugar solution and kept in dark for 1, 5, 12, 24, & 48hrs (T1, T2, T3, T4 &T5 respectively). After each time interval, cages were exposed to sunlight for 3hrs (11am to 2pm), and then transferred to the lab conditions to evaluate viability after one week.

Chlorophyllin concentrations residue in bee body: Bees were frozen after feeding on Cu or Mg-chlorophyllin and homogenized with a polythron in 3ml of 2% sodium dodecyle sulfate (SDS). Suspension was centrifuged at 3000 rpm for 10min at room temperature. Pellet was discarded, and supernatant aliquots were 10 fold diluted by SDS. Chlorophyllin concentration was measured by the absorption spectrum using Perkin Elmer spectrophotometer Lambda 45. Chlorophyllin relative concentration was determined by optical density compared to control.

Two different closed greenhouses were used, with three hives each, one meter diameter water container and cultivated flowering clover plants. First one was a control and the second two were treated ones, containing fresh water mixed with copper chlorophyllin LC_{50}. All were examined on a daily basis, for bees' behavior and mortality percent.

Statistical analysis: Average measurements on multiple aliquots of each sample was used as data analysis. Differences were calculated with two-way ANOVA. Subsequently, means were separated by Tukey's HSD test (a = 0.05).

Results

LC_{50} concentrations of copper chlorophyllin on viability were given (Fig. 1 a, b, c, d, e, & f). Concentrations of 10^{-6}, $3x10^{-6}$, $5x10^{6}$, $7x10^{-6}$, $9x10^{-6}$ & 10^{-5} M/L of Cu-chlorophyllin showed mortalities after 3 to 5 day post exposure respectively. Maximum effect of cu-chlorophyllin on bees was 25% for highest concentration (10^{-5}M/L) after five days light exposure. Dark control and normal control mortality did not exceed 5% & 4 % respectively. Cu-chlorophyllin LC_{50} was $3x10^{-5}$ M/L (Fig. 2).

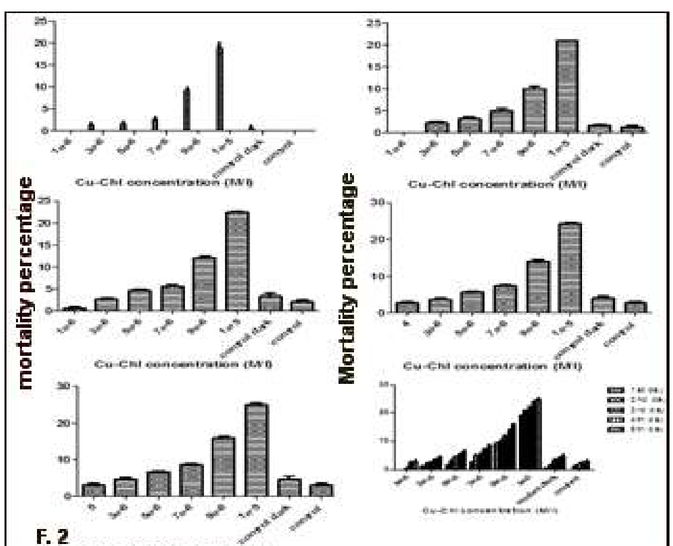

Fig. 2: Effect of 10^{-6}, $3x10^{-6}$, $5x10^{-6}$, $7x10^{-6}$, $9x10^{-6}$ & 10^{-5} M/L of Cu-chlorophyllin on survival % of *Apis mellifers* (20 workers/ concentration) exposed to sun light for 8hrs after feeding on Cu-chlorophyllin ad libitum.

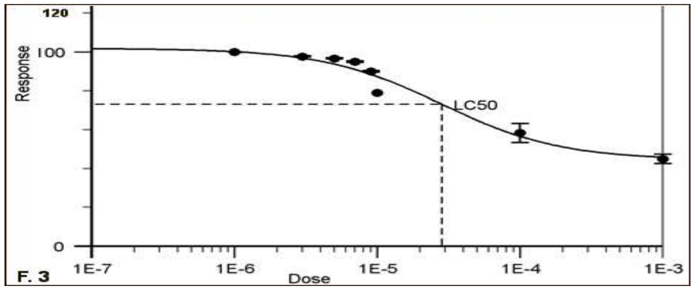

Fig.3: LC50 of Cu-chlorophyllin against honey bee workers.

In Mg-chlorophyllin LC_{50} on viability (10^{-6}, $3x10^{-6}$, $5x10^{-6}$, $7x\ 10^{-6}$, $9x10^{-6}$ & 10^{-5} M/L) on bee workers exposed to sun light for 8hrs (Fig. 3a, b, c, d, e, & f) showed mortality from 1st to 5th day post exposure respectively. Mg-chlorophyllin maximum effect was 9% mortality at high conc. (10^{-5} M/L), five days post exposure. Lowest one decreased in descending order of concentrations. Dark control and normal control mortality not exceed 6% &4% respectively and Mg-chlorophyllin $LC_{50}$was $3x10^{-3}$ M/L (Fig. 4).

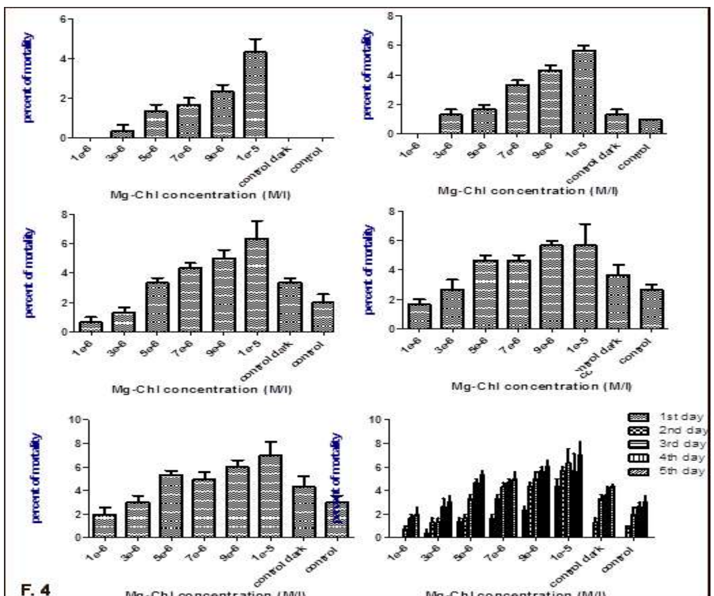

Fig. 4: Effect of 10^{-6} , $3x10^{-6}$, $5x10^{-6}$, $7x10^{-6}$, $9x10^{-6}$ & 10^{-5} M/L of Mg-chlorophyllin on survival% of *Apis mellifers* (20 workers/ concentration) exposed to sun light for 8hrs after feeding on Mg-chlorophyllin ad libitum.

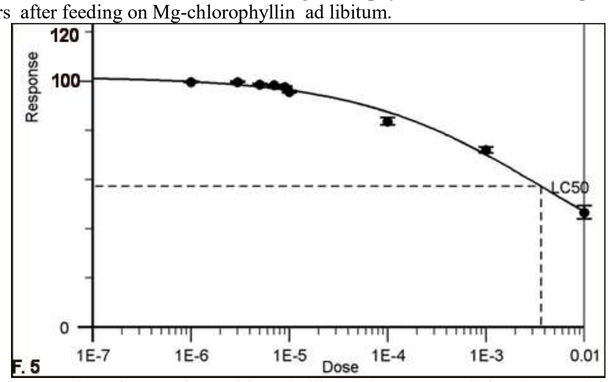

Fig. 5: Value of LC_{50} of Mg-chlorophyllin against *Apis mellifera* bee workers.

Effect of exposure sunlight on viability of honey, one optimum concentration of chlorophyllin ($5x\ 10^{-6}$ M/L) was used (Fig. 6a & b). Data showed the percentage of mortality as a function of different exposure times after 1, 3 & 6 hours for Mg-chlorophyllin and

Cu-chlorophyllin respectively. The percentage of mortality increased significantly as increasing time exposure. The maximum mortality rate after 6 hours exposure time was 7% and 10% for Mg-chlorophyllin and Cu-chlorophyllin respectively in the first monitoring day post light exposure. This mortality rate increased at the 5th monitoring day to be 14% & 21% for mg-chlorophyllin and cu-chlorophyllin respectively. Other exposure times caused lesser mortality rates comparing with the control experiment.

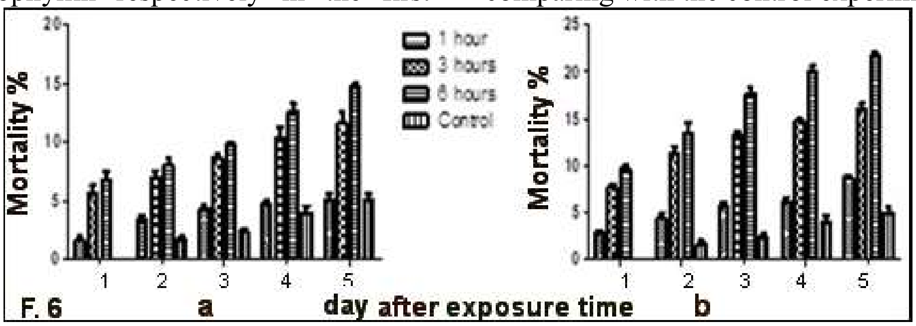

Fig. 6: Effect of different exposure times (1, 3, & 6hrs) of sunlight on % of mortality of *Apis mellifers* (20 workers/ each exposure time) after feeding on $5x10^{-6}$ M/L of (a) Mg-chlorophyllin and (b) Cu-chlorophyllin.

Accumulation dynamic of Cu-chlorophyllin and Mg-chlorophyllin by bees: Behavior of feeding agents accumulation appeared different in used of 2 derivatives of chlorophyllin. In case of Cu-chlorophyllin accumulation, there was sharp accumulation in first 3hrs of feeding (0.3un). In the next 9hrs the accumulation rate decreased to one third, and the accumulation rate increased again against feeding time (Figs. 7 & 8).

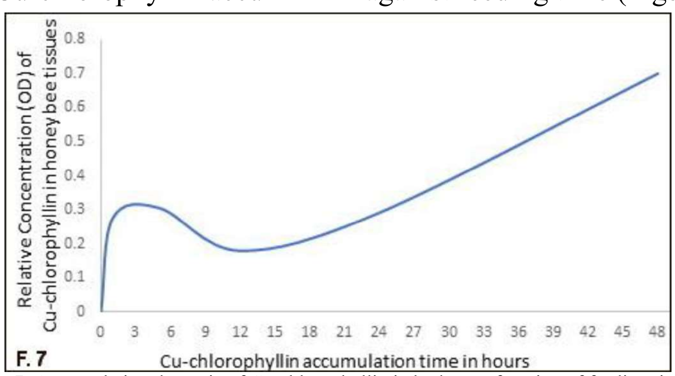

Fig.7: Accumulation dynamic of Cu-chlorophyllin in body as a function of feeding time.

The Mg-chlorophyllin accumulation gave high accumulation rate in the first hr. and from the10th to 20th hrs., without increased up to the end of the experimental.

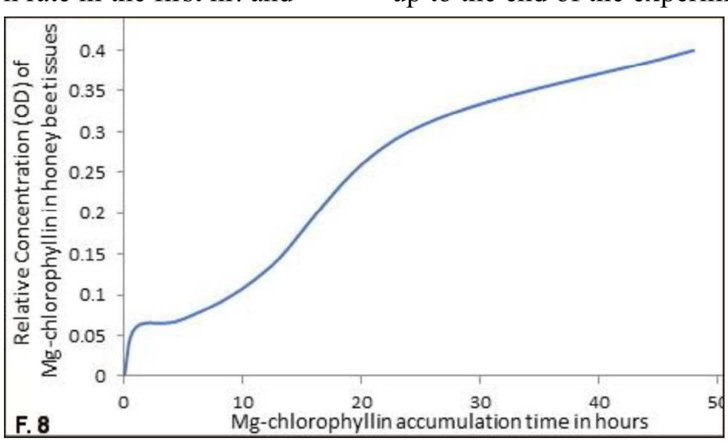

Fig. 8: Accumulation dynamic of Mg-chlorophyllin in honey bee body as a function of feeding time.

Dynamic of Cu-chlorophyllin and Mg-chlorophyllin was released by honey bee respectively in time intervals after replacing chlorophyllin food by sugar solution. The release of chlorophyllin derivatives was in slow rate in the first 12hrs from chlorophyllin food removal. After 12hrs there was a significantly high increase in rate of release of chlorophyllin derivatives. This high release next hours to reach to zero and 0.1un concentration for cu-chlorophyllin and mg-chlorophyllin respectively (Figs. 9 & 10).

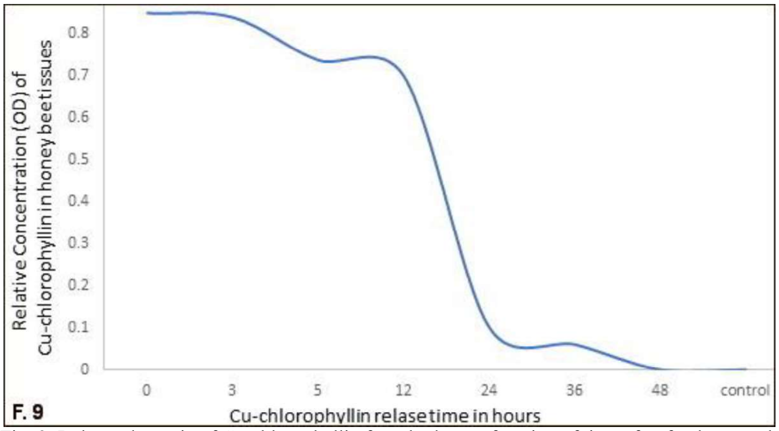

Fig. 9: Release dynamic of Cu-chlorophyllin from body as a function of time after food removal.

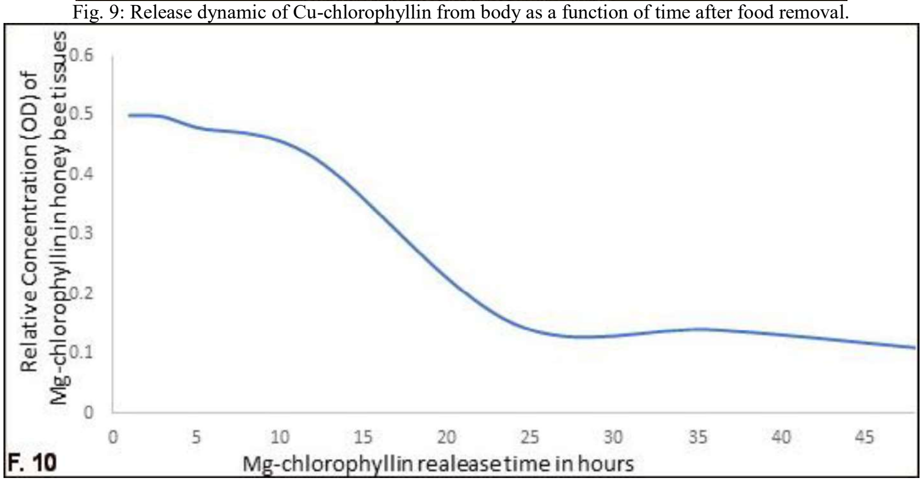

Fig. 10: Release dynamic of Mg-chlorophyllin from body as a function of time after food removal.

Remained chlorophyllin on bee mortality when exposed to sunlight after 1, 5, 12, 24 & 48hrs, when replaced chlorophyllin food by normal sugar bait, reduced percentage as a function of elapsed time increase in all days. Maximum mortality rate was after 1, 5, 12, 24 & 48hrs elapsed time were 24%, 28%, 25%, 20%, 10% respectively (Fig. 11).

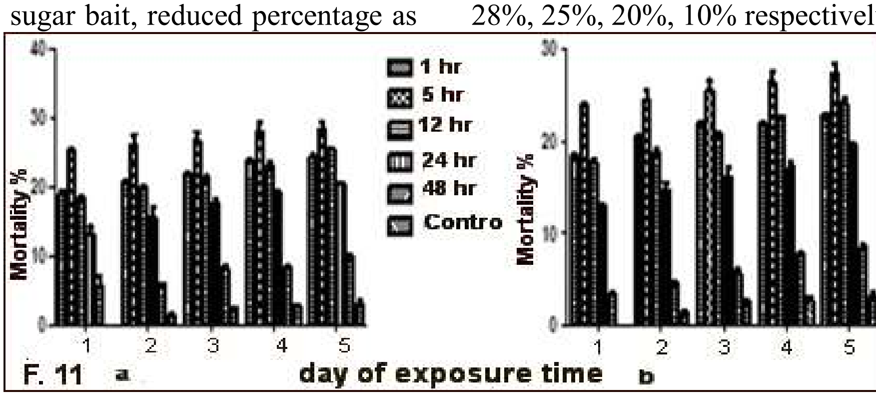

Fig. 11: Effect of elapsed time of cu-chlorophyllin (a) & mg-chlorophyllin (b) release on percentage of honey bee mortality.

There was insignificant difference between mortality rates of hives exposed to water (control) and those exposed to water treated by chlorophyllin derivative (treated) in 30 days monitoring post experiment onset. In control ones, number of dead bees in 1st, 5th, 10th, 15th, 20th, 25th, & 30th day were 50, 70, 50, 80, 50, 70 & 30 respectively. In treated hives, dead bees were 60, 50, 60, 70, 40, 60 & 40 respectively (Fig. 12).

Discussion

Chlorophyll derivatives are used as photopesticides against medical and agricultural pests (Dokmak *et al,* 2021).

In the present study, different concentrations of Cu-chlorophyllin and Mg-chlorophyllin were used in honey bee's feeding bait to determine the effective LC_{50} (50%) and LC_{100} (100%) mortality concentrations. After that it was easy to find the effect overlap points between effective concentration of Cu-chlorophyllin and Mg-chlorophyllin on honey bees and the effective concentrations range used for noxious insects control (ex. mosquito's larvae). As the efficiency of photochemical reaction of chlorophyllin derivatives depends on two main factors, chlorophyll derivative concentration and exposure time, the experiment protocol of this study considered these main factors and related secondary factors. The applied and effective concentration of chlorophyll based insecticide to control medical or agriculture insects is ranged between 10^{-6} up to 7x 10^{-6} M/L (Abdel-Naby, 2019).

In the present study, the maximum mortality rate in bees did not exceed 10% within a range of 10^{-6} -7x 10^{-6} M/L and reached 25% at the highest concentration used 10^{-5} M/L, and in control the mortality percentage was 5%. Also, corresponding curves showed the LC_{50} for both of Cu-chlorophyllin and Mg-chlorophyllin, the effective photo-pesticides. LC_{50} of Mg-chlorophyllin both derivatives were less toxic than Cu-chlorophyllin on the viability of bees.

Bee workers must daily supply their colony with a balance of pollen and nectar to sustain optimal colony development exposed to direct sunlight (Huang *et al,* 1992).

The present study, evaluated the effect of exposure time on honey bee workers when exposed to the Cu-chlorophyllin and Mg-chlorophyllin photo-pesticides in nature. The three exposure periods were applied (1, 3 & 6hrs) and an optimum concentration was used ($5x10^{-6}$ M/L). Maximum mortality rate was 14% & 21% for Mg-chlorophyllin and Cu-chlorophyllin respectively. So, the sunlight as one of the main factors of photochemical reaction did not exceed the safety margin needed to apply these pesticides safely in the field of honey bees hives. Oral chlorophyllin may cause false positive. Generally, oral preparations of sodium copper chlorophyllin (or chlorophyllin copper complex) are available in supplements and as an over-the-counter drug (Derifil® generic name: chlorophyllin) used to reduce odor from colostomies or ileostomies or to reduce fecal odor due to in-continence and sodium copper chlorophyllin was used as a color additive in foods, drugs, and cosmetics (Bohn *et al*, 2004). Besides, the relation between existence of Cu-chlorophyllin and Mg-chlorophyllin in honey bee food medium was digested in human intestine (Ferruzzi *et al,* 2002).

In the present study, bee showed different behaviors in accumulation dynamic of the chlorophyllin derivatives. Cu-chlorophyllin accumulation rate jumped in the first 3hrs and in next 9hrs decreased by one third then the accumulation increased in a constant rate against feeding time. In case of Mg-chlorophyllin accumulation, the high accumulation rate was two times intervals in the hour from 10th up to 20th hour. After the 20 hours feeding, the accumulation has constant increase up to the end of experiment. The dynamics of Cu-chlorophyllin and Mg-chlorophyllin release by honey bee respectively in time intervals after replacing chlorophyllin food by sugar solution. Comparing the data in the accumulation rate experiment and release rate experiments for both of Cu-chlorophyllin, and Mg-chlorophyllin, bees showed high efficiency in getting rid of the accumulated chlorophyllin derivatives few days after feeding and 48 hours were enough for the honey bee to release all the accumulated chlorophyllin derivatives, which varied in mortality rates during exposure times. Maximum mortality rate did not exceed 14% and 21% for Mg-chlorophyllin and Cu-chlorphyllin-respectively.

In the present study, the mortality rate decreased whenever bees were exposed to sunlight. This explores the effect of remaining mg-chlorophyllin and cu-chlorophyllin on percentage of honey bee's mortality when exposed to sunlight after time intervals (1, 5, 12, 24 & 48hrs) from replacing chlorophyllin food by normal sugar bait, attributed to high efficiency of bee release dynamic that decreased the photooxidation stress of mg-chlorophyllin and cu-chlorophyllin by reducing accumulated concentration in 2 days. This agreed with Krasnoff *et al.* (1994).

In the present study, both chlorophyllin and its conjugated metal exert an oxidative stress for bees, which led to toxicity, which agreed with Pardini, (1995); Ercal *et al,* (2001) and Wu *et al.* (2016). No doubt, chlorophyllin increased oxidative stress on the bee queen and workers caused risky changes on bee colony health and longevity (Alaux *et al,* 2011). In the present study, in field experiment no significant difference was found between mortality rates in treated and control hives. The real activity of honey bee workers outside the hive and the probability of the insects to be exposed to sunlight and treated water were less than what was expected from the laboratory testing.

Conclusion

Honey produced by bee is widely used across time and space. LC_{50} of Mg-chlorophyllin derivatives was less toxic than Cu-chlorophyllin on bee's viability exposed to same experimental conditions.

Accumulation of chlorophyllin derivatives in bee body was reduced by antioxidant system decreased photo-oxidation stress of Cu-chlorophyllin and Mg-chlorophyllin.

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Explanation of figure

Fig. 12: Dead bees in hive closed to chlorophyllin derivative free water basin (control) and bees hive closed to water mixed with chlorophyllin derivative (10^{-5} M/L) basin (treated).