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Original Article

Using Citronella to Protect Bees (honeybee *Apis mellifera* L.) from certain Insecticides and Their Nano Formulations

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(With 9 figures)

Abstract

Experiments were performed investigating citronella (*Cymbopogon winterianus* Jowitt) as a repellent to honeybee *Apis mellifera* (L.) (Hymenoptera: Apidae) in Egypt, it was conducted in laboratory in the Department of Entomology and Pesticides Science, Faculty of Agriculture, Cairo University, to check long-term survival of honeybee when exposed to different nano insecticides alone or combined with citronella at the same examination box for each. In this study, we used a modeling approach regarding survival data of caged worker bees under chronic exposure to four insecticides (Chloropyrophos, Nano-chloropyrophos Imidacloprid, Nano-Imidacloprid) each of them was supplemented in a box alone and in combination with citronella. Having three replicates and five concentrations (100, 200, 300, 400 and 500 ppm). Laboratory bioassay of these insecticides showed that chloropyrophos and nano chloropyrophos were the most toxic at their high dose (500 ppm) with LT_{50} of 120.98 and 122.02 followed by 132.14 and 136.5 minutes for Imidacloprid and Nano-Imidacloprid, respectively. No consumption occurred by bees to mixed sugar syrup with insecticides in all treatments when citronella was added. These data highly recommended that adding citronella is very effective when nicotinoid pesticides are used to longevity honeybee life and keep bee safe.

Keywords: honeybee, Nano-Chlorpyrifos, Nano-Imidacloprid, citronela, toxicity.

Uso da citronela para proteger as abelhas (*Apis mellifera* L.) de certos inseticidas e suas nanoformulações

Resumo

Foram realizados experimentos para investigar a citronela (*Cymbopogon winterianus* Jowitt) como repelente de abelhas *Apis mellifera* (L.) (Hymenoptera: Apidae) no Egito, conduzidos no laboratório do Departamento de Entomologia e Ciência de Pesticidas, da Faculdade de Agricultura, da Universidade do Cairo, e verificar a sobrevivência a longo prazo das abelhas quando expostas a diferentes nanoinseticidas isoladamente ou combinados com citronela na mesma caixa de exame para cada um. Neste estudo, usamos uma abordagem de modelagem em relação aos dados de sobrevivência de abelhas operárias enjauladas sob exposição crônica a quatro inseticidas (clorpirifós, nanoclorpirifós, imidaclopride e nanoimidaclopride), e cada um deles foi suplementado em uma caixa e em combinação com citronela, tendo três repetições e cinco concentrações (100, 200, 300, 400 e 500 ppm). O bioensaio em laboratório desses inseticidas mostrou que clorpirifós e nanoclorpirifós foram os mais tóxicos em altas doses (500 ppm) com LT50 de 120,98 e 122,02, seguidos por 132,14 e 136,5 minutos para imidaclopride e nanoimidaclopride, respectivamente. Não houve consumo pelas abelhas do xarope de açúcar misto com inseticidas em todos os tratamentos quando a citronela foi adicionada. Esses dados recomendam a adição de citronela, sendo muito eficaz quando pesticidas nicotinoides são utilizados para longevidade das abelhas e para mantê-las seguras.

Palavras-chave: abelha, Nanoclorpirifós, Nanoimidaclopride, citronela, toxicidade.

1. Introduction

A lot of reports suggesting that exposure to citronella odor repel honeybees (Malerbo-Souza and Nogueira-Couto 2004; Abramson et al., 1999a, Abramson and Aquino 2002a, b; Aquino et al., 2004). In addition to helping ensure a diverse supply of food for humans, pollination plays a critical role in providing the basis for essential ecosystem productivity and services (Kevan et al., 1990; Kevan, 1999., Antonini et al., 2006). There is concern about potential adverse effects of pesticides on pollinators (EFSA, 2012; National Research Council, 2007). Considerable effort has been directed at finding a honeybee repellent because of public safety issues (Abramson et al. 1997a), the possibility of providing researchers interested in reducing the effects of harmful agrochemicals (Atkins Jr. et al., 1975a, 1975b;

Mayer, 1997). There are several studies in the literature suggesting other repellent to honeybees (Blum et al., 1978; Free, 1987; Free et al., 1989). Impact of pesticides as a bioassay to screen for adulterated honey was assessed (Abramson et al.; 1999b; Abramson et al., 2006). In this report, the ability of citronella odor to repel honeybee under laboratory conditions was tested. We believe this approach can serve as a model for testing repellents in honeybees. However, the number of managed honeybee colonies has declined in Europe and the United States (Dainat et al., 2011; vanEngelsdorp et al., 2011) due to multiple factors (Dainat et al., 2012; van Lexmond et al., 2015). Recently, attention has focused on neonicotinoid pesticides like imidacloprid, which can impair individual and colony fitness at even sublethal doses (Desneux et al., 2007; Goulson, 2013; Henry et al., 2012; Sanchez-Bayo, 2011). Imidacloprid is a systemic insecticide that spreads throughout all plant tissues and is found in nectar and pollen at concentrations up to 50 ppm (Goulson, 2013). Imidacloprid can also be found in water with 8% (Johnson and Pettis, 2014). Chlorpyrifos is an organophosphorus insecticide control a wide variety of foliage- and soil-borne insect pests on a variety of food and feed crops (Solomon et al., 2014). Rising interest occurred recently with nanopesticides. Nano-imidacloprid has been used to control several insects (Sabbour, 2015; Assemi et al., 2014). This study refers to using citronella could be very effective especially when we use nicotinoides pesticides, in order to repel bee and keep them safe.

2. Material and Methods

2.1. Insect

Honeybee *Apis mellifera* workers obtained from apiary at Faculty of agriculture, Cairo University.

Preparation of Nano Pesticides

Nanochlorpyrifos and nanoimidacloprid were both prepared at Nano Tech Company, Dreamland, 6th October City, Egypt according to the method described by Guan et al. (2008). The Fourier Transform Technique (for Advanced Readers) Fourier Transform type (Mac Company) was used; the technique was according to (Griffith and Fuller, 1982).

2.2. TEM Analysis

The shape and size of both nano chlorpyrifos and nano imidacloprid were analysed and observed under Transmission Electron Microscope (TEM), JEOL model 3010 Philips CM-200, Japan operated at 120 kv, they were prepared according to Parveen et al. (2014).

2.3. Bioassay

Toxicity was studied with special wooden boxes of diameters (75 cm width and 80 cm height surrounded with metal net from all sides). In this method, we supplied each cage with two petri dishes 10 cm diameter, each one contained 5ml of each insecticide or insecticide with citronella with recommended repel concentration. Five honeybees' workers were introduced, along with one control box free of any pesticides but it containing distilled water and sugar (1:1). This treatment was replicated three times.

Also, mortality percentage was determined after 30, 60, 90, 120 seconds and so on. Honeybee workers were kept in cage for very shorter periods of time (up to 24 hours). Methodology for assay was developed in the lab, so there is no reference in this regard. The boxes were placed at temperature 25 ± 10 ºC and 50 ± 5% humidity. Food consummation was determined through measurements of syrup after 2 hours from each petri dish. Besides our team designed direction box attitude (Figure 1) where we join tow boxes with cylindrical bridge with open passage (path) from the top to release bees, one of the boxes contained Nano-Imidacloprid 500 ppm and the other one contained the same insecticide with citronella and bees released in the middle to the bridge and let to choose any direction freely where to go.

2.4. Statistical analysis

LD50 values were estimated by plotting log dose versus probit plus five mortality (Sokal and Rohlf, 1995; Finney, 1971; Microsoft Excel, 1997).

3. Results and Discussion

The sudden disappearance of honeybee from hives has been called "Disappearing Disease" (Wilson and Menapace, 1979). It was noted and referred to as Colony Collapse Disorder (CCD) by researcher, also corresponded to increased use of neonicotinoid pesticides (Johnson et al., 2010; Cresswell et al., 2012). This has led to speculation that there is a causative relationship between increasing using by neonicotinoids and widely decline in bee populations (Suryanarayanan, 2013). However, it is important to look at all the variables associated with CCD. Malerbo-Souza and Nogueira-Couto (2004) found that spraying citronella temporary decrease in the number of bees visiting a test site (Harpaz and Lensky, 1959). It has been known for some time that honeybee learn to associate a floral scent with (Menzel et al., 1993). It is entirely likely that the odor of the test repellent is the reason of this. (Abramson et al., 1996, Silva and Rebêlo, 2002). Our belief on ongoing research is increasing our understanding of the impact of these types of pesticides on bees. For now, the best recommendation is to carefully follow the product label,

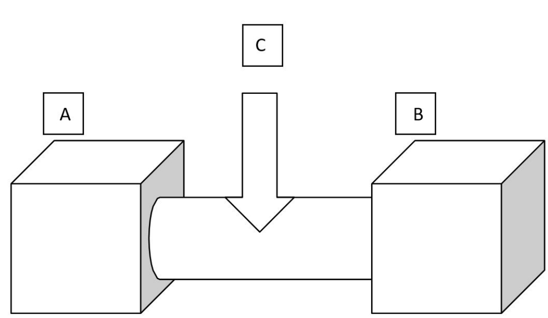


Figure 1. Cage to investigate bee food preferences where A contains pesticide alone and B contains Pesticide and citronella mixed together and C a passage or path where bee let go free to A or B

be judicious in your application, and avoid applying any insecticide product, if necessary you should it is useful to use citronella as a repellent so you don't affect the bees.

3.1. Food consumption and wheight

All treatments were detected for syrup consumption and preference of bee to eat, and it was found that all treatments concluded citronella was very detritus, where it scored zero consumption in comparison to other treatments where bee preferred to feed in control treatments followed by Imidacloprid, Chloropyrophos, Nano-Imidacloprid and Nano-Chloropyrophos; respectively, also in direction cage experiment 97.3% of total released bees moved towards the box containing only pesticide avoiding the other box that contain pesticide mixed with citronella.

3.2. Legs Shrinkage

The importance of examine this parameter; attributed to that Imidacloprid is considered neurotoxin where it affects mostly the synapsis in CNS of honeybee. Results of Nano-Imidacloprid showed that highly affected on worker legs where it recorded 100.00, 90.00, 100.00, 89.47 and 91.66% with nano Imidacloprid 100, 200, 300, 400 and 500 ppm, respectively. Where it gave 63.63% with Imidacloprid and 100.00 with Chloropyrifos. These ratio decreases significantly where Nano-chloropyrophos was examined, it gave 0.00, 75.00, 63.15, 58.33 and 100.00% with 100, 200, 300, 400 and 500 ppm concentrations, respectively. On the other hand, for sugar syrup that formulated from distilled water, data obtained showed that shrinkage in legs was limited where it gave 11.11% only, (Figure 2)

3.3. Circulation Degree of the Body

Nano-Imidacloprid results showed that highly affected on body balling of honeybee workers where it recorded 88.00, 100.00, 100.0, 90.00 and 91.00% with Nano-Imidacloprid 100, 200, 300, 400 and 500 ppm, respectively. Where it gave 100% with both Imidacloprid and Chloropyrifos, this ratio decreased significantly where Nano- Chloropyrophos was examined; it gave 10.00, 62.50, 57.14, 78.94 and 83.33% with 100, 200, 300, 400 and 500 ppm concentrations, respectively. On the other hand, for syrup formulated from distilled water and sugar there were no shortage (balling) occurred at all worker members (Figure 3).

3.4. Abdomen shortage

Imidacloprid can have delayed toxic effects in bees, particularly in chronic exposure (Rondeau et al., 2014). Their degradation was toxic to bees (Goulson, 2013). Imidacloprid is insecticidal because it is an agonist of insect nicotinic acetylcholine receptors (nAChRs) (Gauthier, 2010), which are found in honeybee (Jones et al., 2005) and play a role in bee learning (Dacher and Gauthier, 2008). Honeybee nAChRs play an important role in cholinergic neural signaling, and bees fed sublethal doses of imidacloprid down regulate nAChRs in their brains (Zhou et al., 2014). Imidacloprid has a wide variety of effects: brain cell death (Wu et al., 2014), impaired foraging (Schneider et al., 2012, decreased food uptake (Ramirez-Romero et al., 2005), diminished hive entrance activity (Decourtye et al., 2004a), reduced motor function (Lambin et al., 2001; Williamson and Wright, 2013), impaired visual learning (Han et al., 2010), decreased predator avoidance (Araújo et al., 2004), impaired navigation to the nest (Fischer et al., 2014) flowers (Dukas and Morse, 2003; Morse and Nowogrodzki, 1990; Reader et al., 2006). This is a complementary parameter where it gave obvious picture on situation of muscles, which really were affected, that in all treatments no significant effects were found in abdomen shortage (Figure 4).

3.5. Wing horizontal

The normal attitude to have 0.00% wing horizontal manner. As it is illustrated in Figure 5 where this parameter gave 0.00% in case of syrup free of pesticide, where it

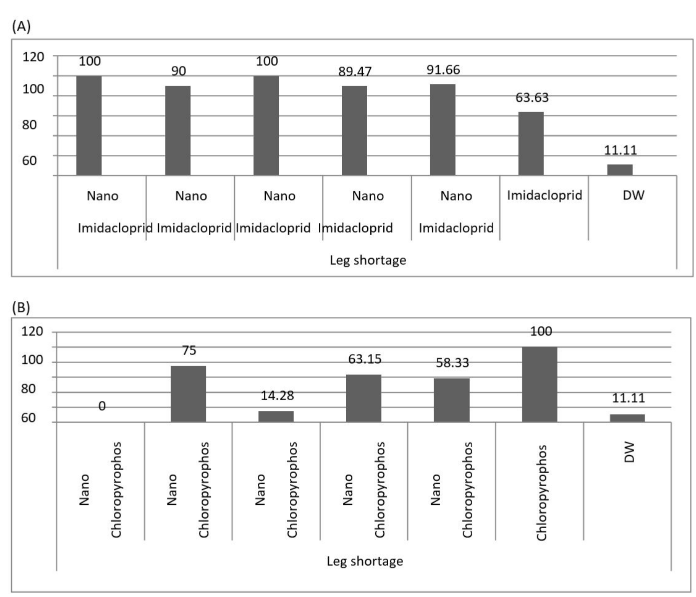


Figure 2. Leg shortage in honey bee workers caused by (A) Imidacloprid and nano Imidacloprid and (B) Chloropyrophos and nano chloropyrophos.

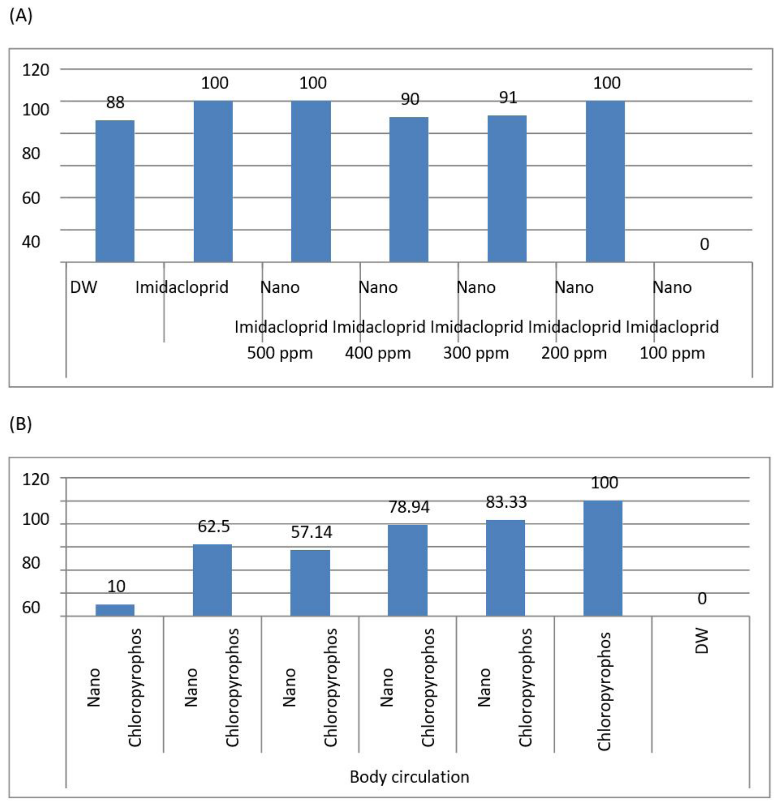


Figure 3. Body circulation in honey bee workers caused by (A) Imidacloprid and nano Imidacloprid and (B) Chloropyrophos and nano chloropyrophos.

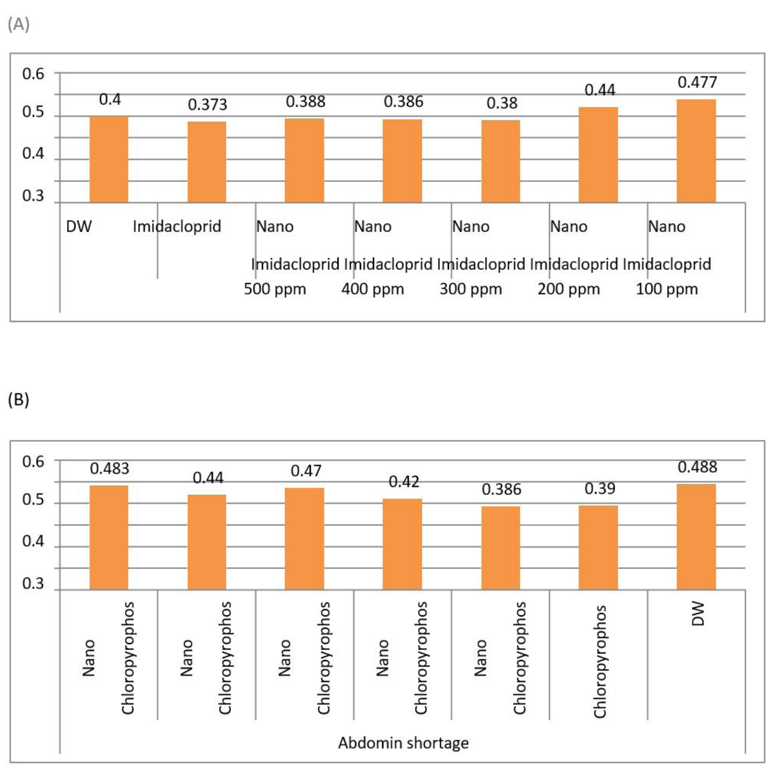


Figure 4. Abdomen shortage in honey bee workers caused by (A) Imidacloprid and nano Imidacloprid and (B) Chloropyrophos and nano chloropyrophos.

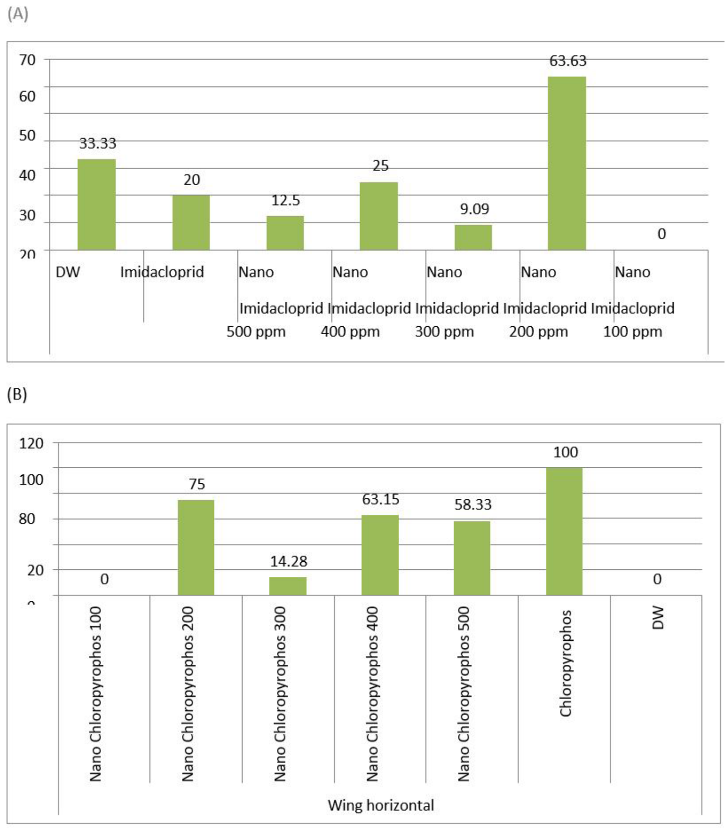


Figure 5. Wing horizontal in honey bee workers caused by (A) Imidacloprid and nano Imidacloprid and (B) Chloropyrophos and nano chloropyrophos.

gave 63.63% with Imidacloprid and 33.33, 20.00, 12.5, 25.0, and 9.09% with Nano- Imidacloprid concentrations (100, 200, 300, 400 and 500, respectively). In contrast, this ratio increased gradually with chloropyrophos where it gave 100% with chloropyrophos, and 0.00, 75.00, 14.28 and 63.15 and 58.33% with 100, 200, 300, 400 and 500 ppm of nano-chloropyrophos concentrations, respectively, (Figure 5).

3.6. The effect on mouth parts

This parameter showed highly killing speed where bee dies through having syrup quickly, Figure 6 shows zero % with syrup free of any pesticides. Whereas it was greatly increased with the lowered concentrations of Nano-Imidacloprid, where it recorded 88.88, 80.00, 50.00, 65.00 and 66.66% with concentrations 100, 200, 300, 400 and 500 ppm, respectively. And 36.36% with Imidacloprid.

This effect decreased significantly with chloropyrophos which scored 18.18% only, and its nano form recorded 0.00, 75.00, 14.28, 63.15 and 58.33% with 100, 200, 300, 400 and 500 ppm, respectively.

3.7. The fourier transform technique

The nano-pesticides were examined for its activity stability according to (Griffith and Fuller, 1982; Griffith and Haseth, 1986; Calvert et al., 1993; Reuter et al., 1998; Johnson et al., 2000). Data given in Figures 7 and 8 revealed that nano-chlorpyrifos almost the same peaks as compared to with chlorpyrifos while nano-imidacloprid peaks almost reduced to a half when compared to active peaks with imidacloprid.

3.8. TEM analysis

Both of imidacloprid and chlorpyrifos nanoparticles revealed nano size where the mean of counted particles were less than 100nm for nano chlorpyrifos (with median range 44.5nm) and nano imidacloprid (with median range 88.7nm) (Figure 9), once nutrition; Respectively. Metabolites of imidacloprid were found in honeybee (Nauen et al., 1998; Decourtye et al., 2004b). Yamamoto et al. (1998) findings suggest that metabolism and detoxification pathways may vary between insect species which can affect insect susceptibility to neonicotinoids. We provide the first evidence that the neonicotinoid, nano-Imidacloprid, chloropyrophos or nano-chloropyrophos can significantly kill honeybee, where nano chloropyrophos, chloropyrophos were less toxic generally in comparison to nano-Imidacloprid and Imidacloprid, and that adding citronella lead to complete avoid to the syrup. We are suggesting that adding citronella and in further investigations field evaluation should be applied and maybe other repellants should be examined.

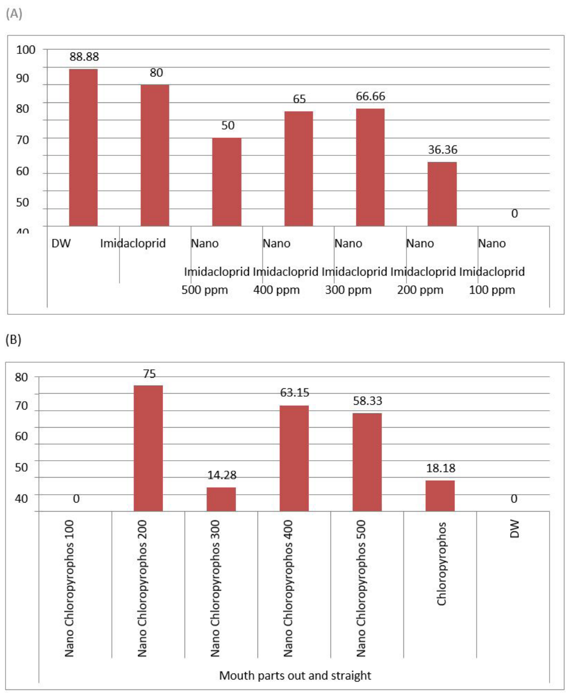


Figure 6. Mouthparts out and straight in honey bee workers caused by (A) Imidacloprid and nano Imidacloprid and (B) Chloropyrophos and nano chloropyrophos.

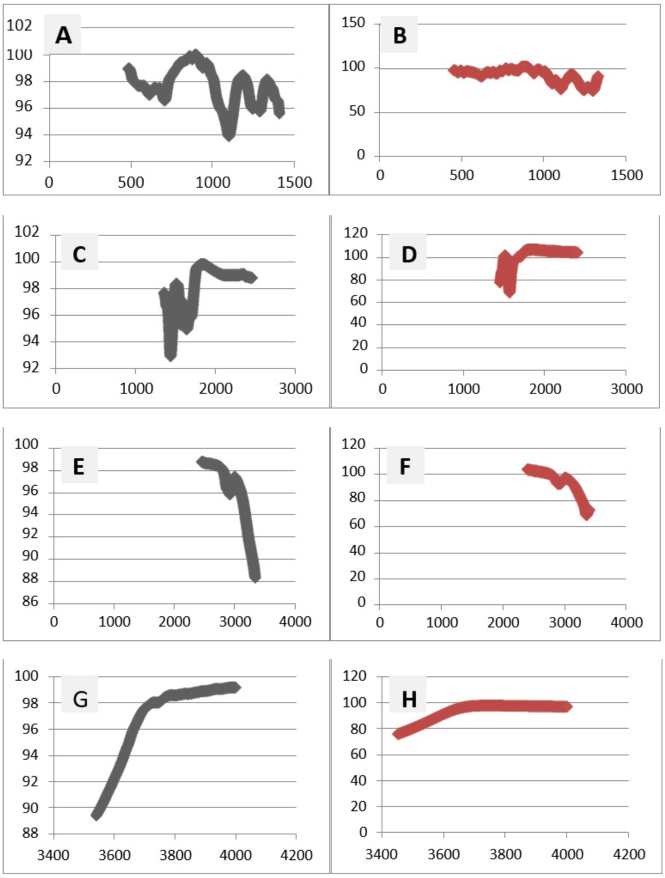


Figure 7. FTIR absorption curves where X represent wave length and Y represent absorption in nanometre (7A, 7C, 7E and 7G) nano Imidacloprid and (7B, 7D, 7F and 7H) represent Imidacloprid

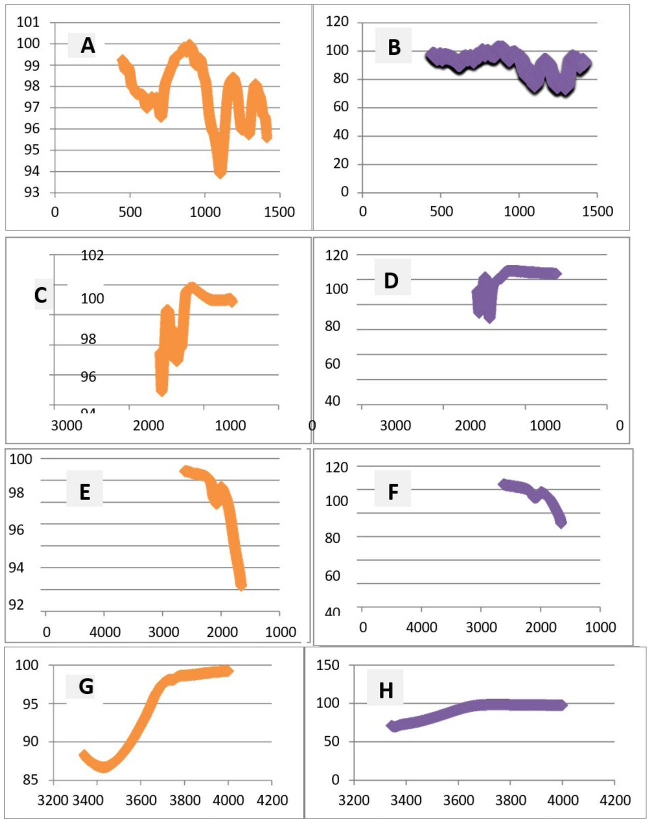
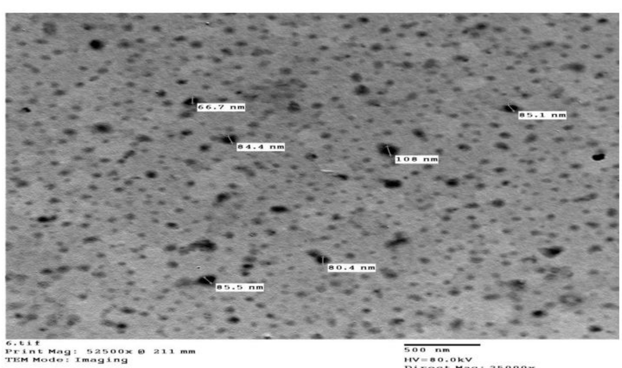
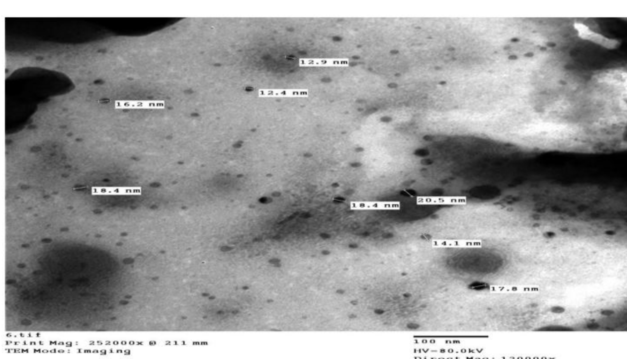


Figure 8. FTIR absorption curves where X represent wave length and Y represent absorption in nanometre (8A,8 C, 8E and8 G) nano Chloropyrophos and (8B,8 D, 8F and 8H) represent Chloropyrophos.



A



B

Figure 9. TEM image showing synthesized A, Imidacloprid nano particles and B, chloropyrophos nano particles.

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