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Glutathione-S-Transferase Response Towards Imidacloprid in Honeybees (*Apis mellifera* L.)

Hussein M Ali[1]*, Basma Abdel-Aty[2], Walaa El-Sayed[2], Faiza M Mariy[2], Gamal M Hegazy[2]

1- Agricultural Biochemistry Dept, Fac of Agric, Ain Shams Univ, P.O. Box 68, Hadayek Shoubra 11241, Cairo, Egypt

2- Plant Protection Dept, Fac of Agric, Ain Shams Univ, P.O. Box 68, Hadayek Shoubra 11241, Cairo, Egypt

*Corresponding author: hussein_galaleldeen@ agr.asu.edu.eg

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Abstract: Imidacloprid is a systematic neonicotinoid widely used to combat piercing-sucking insects; however, neonicotinoids, despite having low effects on vertebrates, showed high adverse effects on honeybees (*Apis mellifera* L.). Glutathione-S-transferases (GST) are an important constituent of the defense system in detoxifying invading chemicals. This work examines the effects of imidacloprid on GST activity *in vivo* and *in vitro* conditions. Results revealed that there are two responses of GST activity toward IMI treatment; first, stimulation of enzyme activity to combat and detoxify the insecticide. Second, direct inhibition of GST, which is confirmed by the *in vitro* inhibition with IC_{50} 887.42 ppm. At a short exposure time to IMI (2 hours) at an IMI concentration of 0.35 ppm, the enzyme was stimulated up to 113% while increasing exposure time or IMI concentration, the inhibition effect dominates.

1 Introduction

The search for effective insecticides against various insects and meanwhile with minimal adverse effects on non-targeted organisms is a continuous process as insects continue to develop their resistance to the applied agrochemicals. These efforts require detailed knowledge of resistance sources and finding differences among organisms to achieve better selectivity.

Glutathione-S-transferases (GST) are a family of enzymes that play a crucial role in the resistance of almost all organisms against both endogenous substrates and exogenous xenobiotics. They catalyze the nucleophilic attack of glutathione toward electrophilic compounds converting them to water-soluble easily excreted substances (Ata and Udenigwe 2008). Electrophilic substrates can be organophosphorus pesticides (Fang 2012), chlorinated compounds e.g. DDT and lindane (Enayati et al 2005), oxirane rings (epoxides), halogenated aromatic rings with strong electron-withdrawing groups e.g. chloro-2,4-dinitrobenzene (CDNB) or Michael acceptors e.g. unsaturated carbonyl compounds (Zimniak 2007).

Neonicotinoids are the most recently developed insecticide group with imidacloprid being the first one introduced to the market in 1991. Since then, they have globally been the fastest-growing group of insecticides because of their many advantages and wide applications (Nauen and Denholm 2005). Neonicotinoids are systematic, water-soluble insecticides (Schmuck and Lewis 2016) that can be used in seed, soil and foliar

applications against many pests e.g. aphids, whiteflies, and planthoppers (Nauen and Denholm 2005). They are used in smaller amounts than those of other insecticide groups e.g. chlorinated hydrocarbons and organophosphorus insecticides (Buszewski et al 2019). In addition, they did not suffer from resistance development by target insects or cross-resistance to other insecticides (Nauen and Denholm 2005, Schmuck and Lewis 2016) and thus they are effectively used against pests that developed resistance against older insecticides (Schmuck and Lewis 2016). Moreover, neonicotinoids showed high selectivity toward insect acetylcholine receptors, the target bio-receptors, over those of vertebrates (Ihara and Matsuda 2018). Despite all of these advantages, there has been increasing public concern since the 2000s about the adverse effects of neonicotinoids on pollinators, especially on honeybee health and colony loss (Lu et al 2020). Therefore, to understand the immunity responses of honeybee insects toward neonicotinoids, the present study aims to examine the effects of imidacloprid, as the most spread neonicotinoids, on GST activity, as a crucial part of honeybee immunity system, under both *in vivo* and *in vitro* conditions.

2 Materials and Methods

2.1 Chemicals and Bee manipulation

Honeybee workers (*Apis mellifera* L.) were collected from the hives of the apiary of the Faculty of Agriculture, Ain-Shams University and distributed in plastic jars (70 bees per jar) for various treatments then starved for 2 hours before treatments. Insects were fed on **s**ucrose syrup (50%) which contains 0-6.3 ppm imidacloprid (IMI) under the conditions of 28 ±1°C and 70 % RH. The insecticide, imidacloprid (1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine), was obtained (35% SC, chinook) from Shora chemicals, Cairo. The substrates, L-glutathione (GSH) and chlorodinitrobenzene (CDNB), were purchased from Solarbio Life Sciences and LOBA Chemie companies respectively. The chromogen, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), was purchased by E. Merck Darmstadt company.

2.2 Effect of IMI concentration on GST activity *in vivo*

Honeybees were fed on sucrose syrup containing 0-6.3 ppm IMI for four hours. Thirty honeybees of each treatment were cooled at 4 °C for 3 min for anesthetization then the heads were dissected on ice. Frozen heads were homogenized, centrifuged (2000 rpm, 15 min) and extracted using iced 3.0 mL phosphate buffer (pH 7.1, 0.1 M) as described by Badawy et al (2015). GST (EC2.5.1.18) activity was determined by the method of Habig et al (1974) as described by Delkash-Roudsari et al (2022) with some modifications in CDNB and GSH concentrations to ensure reaching the enzyme saturation stage. The cuvette contained phosphate buffer (2130 µL, 0.1M, pH= 6.5), reduced glutathione (350 µL, 60 mM in water), CDNB (420 µL, 10 mM in 60% ethanol) and 100 µL enzyme extract. Absorbance was measured against a blank (containing no enzyme) at 340 nm. Results were expressed as percentages of the control experiment (honeybees fed on sucrose syrup only).

2.3 Mortality level

Mortality level was determined by counting the number of dead honeybees during and up to 72 hours after treatments (OECD 1998), insects with appeared up-normality were counted dead. Corrected mortality was calculated using Abbott's formula (1925), [(%T–%C)/(100–%C)] × 100. Where %T and %C are the percentages of dead insects in treatment and control experiments respectively.

2.4 Effect of IMI exposure period on GST activity

Honeybees were fed on a sucrose syrup containing 0.35 ppm for various periods (0-24 hours); then heads were separated and frozen. GST was extracted and activity was determined as described above.

2.5 Effect of IMI concentration on GST activity *in vitro*

GST enzyme was extracted from 100 honeybee heads with 5 mL phosphate buffer (pH 7.1, 0.1 M) as described above. A cuvette containing the phosphate buffer, 50 µL enzyme extract, 175 µL glutathione aqueous solution (60 mM) and 1000 µL IMI solution with various concentrations in the phosphate buffer was shaken then 210 µL of the substrate CDNB (10 mM) or solvent (blank) was added. The solution with a total volume of 3.0 mL was measured spectrophotometrically at 340 nm. Results were expressed as percentages relative to the control experiment (containing no insecticide).

2.6 Statistical analysis

All data are mean of three determinations. One-way ANOVA was applied for the analysis of variance. LSD test at a *p*-level of 0.05 was used to determine the significance of differences among the means using CoStat (6.451) software. Lethal concentration (LC_{50}) was calculated using nonlinear sigmoidal dose-response regression analysis in Origin 2019b software.

3 Results and Discussion

3.1 GST activity responses toward IMI concentration

Glutathione-S-Transferases have an important role in insect resistance and detoxification of various insecticides (Fang 2012). It was reported that GST showed different responses toward pollutants depending on the type of chemical, target organism and exposure conditions e.g. time, temperature and pH (Domingues et al 2010, Fang 2012, Bhagat et al 2016). To rationalize these variable responses, the effects of IMI on GST activity *in vivo* with various concentrations and periods in *Apis mellifera* L. were examined; results were presented as percentages relative to the control experiment (untreated insects) for better comparison. Insects were exposed to the insecticide for four hours then GST activity was determined. Results presented in **Fig 1** showed the effect of various IMI concentrations on GST activity and indicated that enzyme activity is inhibited (92.05%) at a concentration of 3.5 ppm and then increased with increasing concentrations up to 97.05% at 5.6 ppm. At higher concentration (6.3 ppm), the GST activity is even stimulated to reach 107.50%. This result suggests that exposure to IMI can inhibit GST activity but high IMI concentration stimulate resistance and GST activity that can overcome the inhibitory effect.

3.2 GST activity responses toward IMI exposure periods

Since the previous results suggested that, at certain conditions, IMI may inhibit or stimulate GST activity, it is complementary information to identify the effect of increasing IMI exposure time on enzyme activity. Therefore, insects were exposed to IMI continuously for prolonged periods (0-24 hours) at a concentration of 0.35 ppm. Results in **Fig 2** showed that enzyme activity is first elevated after two hours (113.69%) and then returned to an almost normal level (102.88%) after four hours; afterward, GST activity was gradually inhibited to reach 87.20% after 24 hours. Some similarity was observed previously where IMI at a sublethal dose ($LD_{50}/2$) in *A. mellifera* increased GST constantly during 48 hours while in *A. cerana* the enzyme activity increased in the first two hours then inhibition was observed (Li et al 2017). Fang (2012) also found that insecticides could elevate GST gene expression.

3.3 Effect of IMI on GST activity *in vitro*

To rationalize the GST inhibition at a prolonged exposure period toward IMI, after being stimulated to detoxify the insecticide, GST activity was determined against various IMI concentrations *in vitro*. Results (**Fig 3**) showed that the enzyme inhibition is constantly increased with increasing IMI concentration indicating the presence of a direct inhibitory interaction between GST and IMI. However, the inhibitory effect of IMI on GST is not severe and does not contribute to the observed high sensitivity and mortality level of honeybees. It is known that the effective dose of polyphenols *in vivo* is an order of magnitude higher than that *in vitro* to compensate for the permeability degree through the organism tissues as well as the possible biodegradation under the metabolic conditions e.g. enzymes and pH (Fang and Bhandari 2010, Munin and Edwards-Lévy 2011). However, a high concentration (816 ppm) is required to achieve 48.05% *in vitro* enzyme inhibition (IC_{50} 887.42, IC_{80} 3942.35 ppm) which is even much higher than the concentrations used in the field (175 ppm recommended concentration by the manufacturer) to combat other insects; while only 6.3 ppm IMI achieved 88.68% mortality (EC_{80} 6.24±1.95 ppm). Besides no *in vivo* enzyme inhibition was detected at that concentration (6.3 ppm) but oppositely enzyme stimulation (107.5%) was observed. This observation is consistent with the reported effect of the neonicotinoids on acetylcholine receptors as the main target and cause of insect death (Ihara and Matsuda 2018).

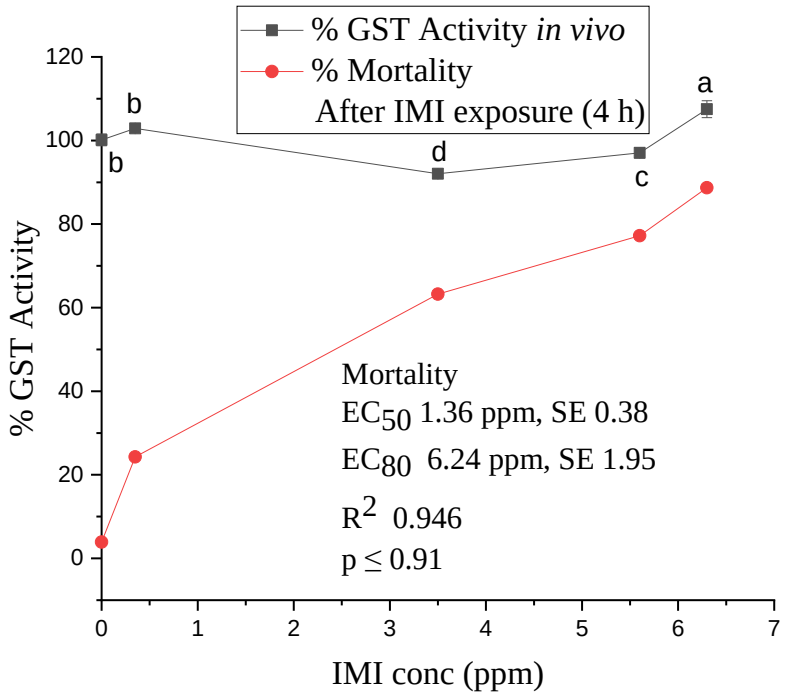

Fig 1. Effects of IMI conc on GST activity and mortality in *Apis mellifera* L. Means with different letters are significantly different at $p \leq 0.05$

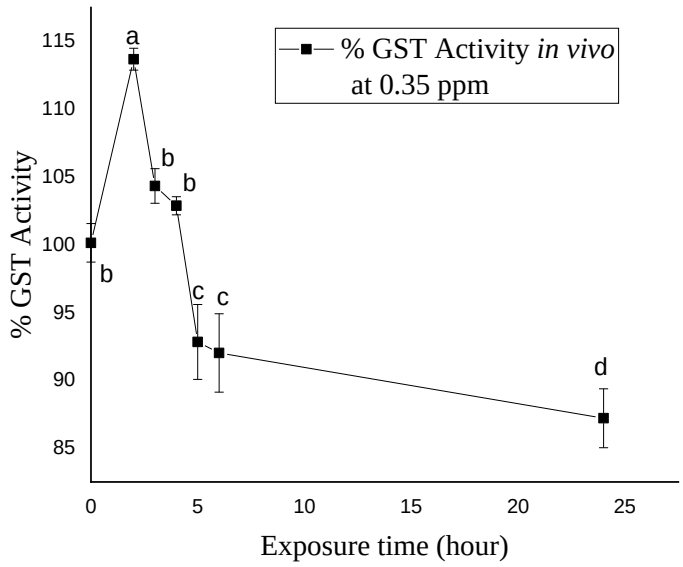

Fig 2. Effect of IMI exposure time on GST activity in *Apis mellifera* L. Means with different letters are significantly different at $p \leq 0.05$

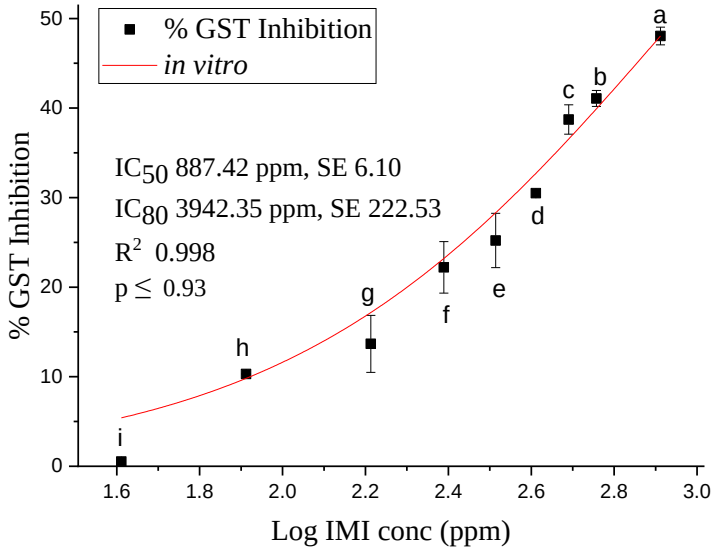

Fig 3. Effect of IMI conc on GST activity *in vitro*. Means with different letters are significantly different at $p \leq 0.05$

4 Conclusion

The present results indicated that GST activity is affected by IMI in two conflicting ways, first, stimulation of enzyme activity to provide higher protection and inhibition by direct interaction with enzyme as revealed by the *in vitro* experiment. Short-term IMI exposure stimulated enzyme activity but prolonging exposure time lowered the protection efficiency as the activity could not afford long-term exposure. In addition, results indicated that enzyme activity is also stimulated by increasing IMI concentrations. On the other hand, mortality level was not correlated with GST activity as it was constantly increased with IMI concentration due to the known interaction with different bio-receptors, i.e. acetylcholine receptors. Accordingly, the work is planned to be extended to cover the IMI interactions with the bio-receptors and the detoxifying GST in target and non-target insects to find out differences that can assist in searching for or designing new neonicotinoids with better selectivity.

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