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Evaluation of DNA damage by insecticides in workers of *Apis mellifera* by comet assay technique

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Abstract

The purpose of the research work was to study the effects of insecticides used in fields on adult *A. mellifera* by determining the DNA damage to the eukaryotic cell's nucleus. Forager worker bees of *A. mellifera* species samples (bees exposed to insecticide/Pesticide in field) were collected from the entrance of the bee hives in district Nankana Sahib, Sialkot, Hafiz Abad, Mandi Bhauddin, Chiniot, Gujranwala, Narowaal, Sragodha, Khishab and Kasure. Hemolymph of these honeybee's samples was ejected by inserting capillary tube between 4th and 5th abdominal segments. Layering of slides was performed using 1% NMP agarose. The single cell gel electrophoresis or comet assay was used to measure damaged DNA in every hemocytes through Epifluorescent microscope. Three parameters i-e Tail Length, Tail DNA and Olive Tail Movement were used to assess the DNA damage caused by insecticides/Pesticides. The results indicated that overall maximum DNA damage was found in honeybees collected from district Khushab. It showed maximum length of comet head (29.05µm), length of tail (26.01µm), length of comet (55.15µm) and tail movement (17.99µm). The minimum genotoxicity was recorded in bees from Nankana sahib area as it had

maximum head DNA length (72.49µm). Olive tail movement from Narowal (7.32µm). It can be concluded that indiscriminate use of insecticides or pesticides in agricultural fields or near honey bees' apiaries caused not only the DNA damage to them but also affects the bee's orientation and other physiological behavior. This in turn impact the strength of the bee colony and honey yield.

Keywords: Comet assay, DNA damage, insecticide, Gene toxicity, Tail length, Head length

Introduction

Honeybees, eusocial flying insects belonging to the genus *Apis*, are crucial for both ecological balance and economic activities. There are nine identified honeybee species globally, most of which inhabit Asia. Among them, the European honeybee (*Apis mellifera*) has been successfully domesticated and holds significant economic value. Its hive products like pollen, honey, and royal jelly support the income of apiarists (Stoner et al., 2019). Globally, honeybees are vital pollinators, contributing over $15 billion annually to the U.S. economy through their pollination activities (Department of Agriculture, US, link). Pollination by honeybees enhances plant biodiversity. However, honeybees face exposure to a range of chemicals used in crop pest control (Winfree et al., 2007; Arena and Sgolastra, 2014). Additionally, they encounter both natural and synthetic acaricides used for treating *Varroa destructor*, a mite that feeds on bee hemolymph (Gashout et al., 2020; Stanimirovic et al., 2022a, b). These chemicals not only weaken bee populations but also impair their hygienic and foraging behaviors. Agrochemical exposure poses significant risks to honeybees in agro-ecosystems, leading to behavioral stresses that can result in population declines. The absence of honeybees can cause a 90% reduction in the yield of some seed, nut crops, and fruits. Compared to many wild bee species, honeybees are versatile, inexpensive, and easy to manage (Arena and Sgolastra, 2014; Irshad et al., 2023). Honeybees are exposed to insecticides through various routes, including soil, pollen (both collected and directly from plants), dandelions, dead and healthy bees, and planter waste products (Magesh et al., 2017; García-Valcárceletal, 2019; Fulton et al., 2019; Glavini et al., 2023). The objective of the current study is to assess DNA damage caused by pesticides and insecticides in *Apis mellifera* using the Comet assay.

Materials and Methodology

Sample collection. Samples of live forager worker *A.mellifera* were collected from different locations of Nankana Sahib, Sheikhupura, Hafizabad, Gujranwala, Mandibhaudin, Narowal, and Sialkot of Punjab province, Pakistan. And guard bees as collected as control. The honeybees were fed with 50% sugar solution as a food source to them till the ejection of hemolymph which was

taken through a capillary tube by creating a slit in the dorsal intersegmental membrane amongst 5 and 6th segment of abdominal. About an average of 15µL of insect blood called hemolymph extracted. This hemolymph of every honeybee was stored separately in an eppendorfs in the refrigerator at 4°C.

Methodology

Method of Singh et al., 1988 was followed to find DNA damages in honey bees. Centrifugation at 1000rpm for 10 minutes was applied on 20 µL of hemolymph and 10µL lymphocytes were normalized in 1mL icy HBSS (/20mMEDTA/10%DMSO). Frozen slides covered with normal melting agarose gel were used to fix 10 µL of cell suspension. Then third coat of 120µL of low melting agarose applied on the top layer and let it hardened for 20 min at 4 °C. At that point, lysing solution composed of NaCl (2.5 M), EDTA (100 mM), , Tris (10mM), DMSO 910%), Triton X-100 (1%) at 4 °C (pH 10) was used for lysis of hemocytes for about 4 hours. . Afterwards, slides were washed for 15 minutes with icy water (3×). Slides placed on a plane gel electrophoresis tray for 20 minutes for ectrophoresis by using electrophoresis buffer of pH 13 consist of 1 mM EDTA and 300 mM NaOH and to unwind the DNA at 20 V and 300 mA for 20 min. Electrophoresis detects DNA damages by separating DNA fragments. Subsequently, Tri's buffer used to defuse the slides were neutralized by tris buffer for 15 min (3×) and then after fixing in ethanol, the slides were dried. Finally, ethidium bromide of concentration 20 µg/mL (60 µL) was used for staining slides for observing cells under a fluorescent microscope for counting. DNA damage depends on the comet tail length. Greater the tail length than head length more will be the DNA damage. In electrophoresis DNA movement depends on the charge to mass ratio. If the fragments are large, it will move slowly otherwise it will move faster. Tail length was measured using an ocular micrometer and according to the equation: Comet length (µm) = total length − diameter of the head Results were statistically analyzed by ANOVA. Mean Comet scores of all groups were also analyzed by LSD to separate significant difference**.**

Results and Discussion

In the current research work mean length of head of damaged DNA was found higher (29.05µm) in Khushab samples followed by Nankana Sahib (22.3µm), Gujranwala (20.45µm), Sargodha (19.35µm), Chiniot (17.55µm), Kasure (17.4µm), Hafiz Abad (15.3µm), Sialkot (13.3µm) and lowest recorded in samples of Mandibha-ud-Din (Fig 1-3). Hayat et al. (2018) had

not described this parameter of genotoxicity. Glavini et al. (2023) tested various concentrations (10, 100, 1000 g/mL) of thymol to determine the genotoxic effect of the drug on cultured *A.mellifera*. Two concentrations (100 and 1000 g/mL) expressed genotoxic effects on genus *Apis* proposing its careful use in beekeeping practices to prevent any negative effects on worker honeybees. Irshad et al. (2023) investigated DNA damage in predatory insects by comet assay method in order to find the impact of environmental factors on the DNA of these insects. The study concluded that by examining the DNA damage, it can be possible to recognize possible threats to their existence and improve strategies for their conservation.

DNA damages were assessed in *A. mellifera* on the basis of length of tail. In this research work mean Comet tail length (LT) of damaged DNA was found higher in district Khushab (26.01 µm). District Narowaal has the second highest mean length of Comet (20.05µm) followed by Kasure (17.75µm), Sargodha (17.45µm), Gujranwala (15.5µm), Mandibha-ud-Din (12.15 µm), Chiniot (12.8µm), Sialkot (12µm), Hafiz Abad (10.6µm) and minimum was measured in district Nankana Sahib (9.05 µm). Hayat et al. (2018) reported that that Comet tail length of *Apis florea* and *A. dorsata* was (15.88µm) and (17.30µm), respectively in insecticides treated zones. District Gujranwala samples had almost same (15.5µm) value of Comet tail length as found for *A. florea* (15.88µm) in insecticides treated areas. Similarly district Sargodha (17.45µm) and Kasure (17.75µm) had the same Comet tail length value (17.30µm) reported for *A. dorsata* in insecticides treated areas (Hayat et al., 2018). Likewise district Khushab had higher Comet tail length (26.01µm) in *A. mellifera* (Fig. 1 & 2) than reported by Hayat (2018) in both *A. florea* and *A. dorstat* honeybees. The samples collected from Narowaal had almost similar length of tail (20.05µm) as recorded *A. dorsata* (19.28µm) in insecticide treated area by (Hayat et al., 2018).

Variation was also found in this study related to the length of Comet (Fig.3); however Hayat et al. (2018) had not explained this parameter. Maximum (55.15µm) average values of length of Comet (LC) was recorded from district Khushab. Second highest value of length of tail was found in Narowal (37.55µm) followed by Sargodha (36.8µm), Gujranwala (35.95µm), Kasure (35.15µm), Nankana Sahib (31.15µm), Chiniot (30.35µm), Hafiz Abad (29.5µm), Sialkot (25.3µm) and the minimum was recorded by Mandibha-ud- Din (22.15µm) samples.

Difference was also found in the average head DNA values in different districts. This current research had shown that that the maximum head damaged DNA was found in district Nankana Sahib (72.49µm), followed by Gujranwala (60.15µm), Chiniot (59.78µm), Hafiz Abad

(54.09µm), Sialkot (51.52µm), Sargodha (46.26µm), Narowaal (43.43µm), Khushab (42.97µm), Kasure (42.53µm) and minimum was shown in district Mandibha-ud-Din (39.19µm) honeybees. Hayat et al. (2018) did not delineate this parameter of DNA genotoxicity.

This research also showed dissimilarities between the tails DNA (TD) in different samples collected from different areas of Punjab. The study revealed that the highest value of mean tail DNA was found in district Khushab (60.84µm) followed by Kasure (57.47µm), Mandibha-ud-Din (57.04µm), Narowaal (56.54 µm), Sargodha (53.74µm), Sialkot (48.51µm), Hafizabad (45.91µm), Chiniot (40.24µm), Gujranwala (39.48µm) and the minimum value of TD was found in district Nankana Sahib (27.31µm). Hayat et al. (2018) also did not describe this parameter of genotoxicity.

In this current research work diversity in term of tail movement was also measured in samples collected from different areas of Punjab. The average tail movement was found maximum (17.99µm) in district Khushab samples, followed by district Narowaal (13.91µm), Kasure (11.24µm), Sargodha (10.79µm), Chiniot (7.66µm), Mandibha-ud-Din (6.98µm), Sialkot (6.73µm), Gujranwala (5.93µm), Hafizabad (5.66µm), Nankana Sahib (2.92µm). Hayat et al. (2018) had not described this parameter of genotoxicity.

This research had analyzed the impacts of pesticides on DNA of adult worker honeybee *Apis mellifera*. This study showed that the average value of olive tail movement was also varying in honeybees' samples collected from different districts. Hayat does also not describe this parameter of research et al. (2018). The highest value of olive tail movement (7.32µm) was recorded in samples of *A. mellifera* collected from district Narowaal, Punjab, Pakistan, followed by Kasure (6.55µm), Sargodha (6.34µm), Khushab (4.56µm), Mandibha-ud-Din (4.22µm), Gujranwala (3.85µm), Sialkot (3.72µm), Chiniot (3.04µm), Nankana Sahib (2.04µm) and the minimum was determined for district Hafiz Abad (2.68µm).

Conclusion

These results suggest significant variation in DNA damage among honeybee populations across different districts, indicating potential environmental or anthropogenic influences. The higher levels of DNA damage observed in Khushab and Nankana Sahib compared to other districts and the comparison with existing data highlight the importance of further investigating the environmental factors contributing to these differences. Additionally, the lack of previous data on

some parameters underscores the need for comprehensive studies to better understand genotoxic impacts on honeybee populations.

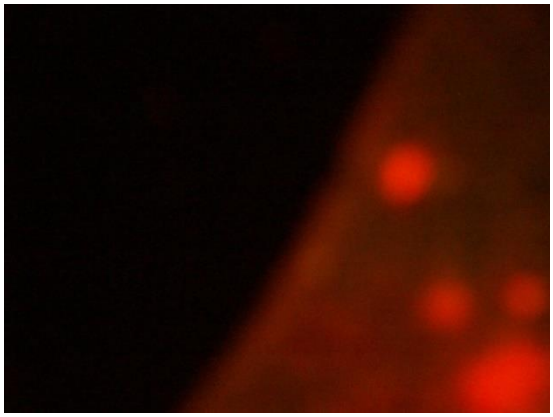

Fig.1. Showing damaged DNA with cell in hemocytes forming a shape of Comet from Khushab.

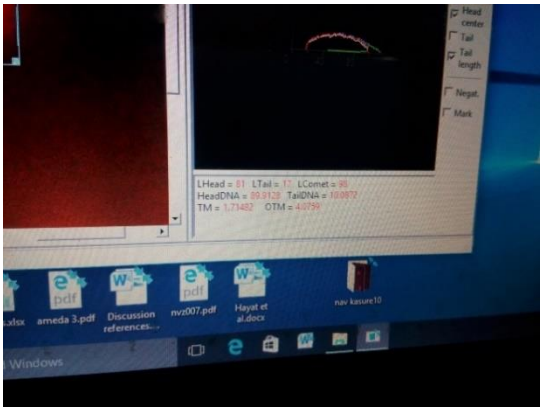

Fig.2. Showing length of comet due to DNA damage from Khushab.

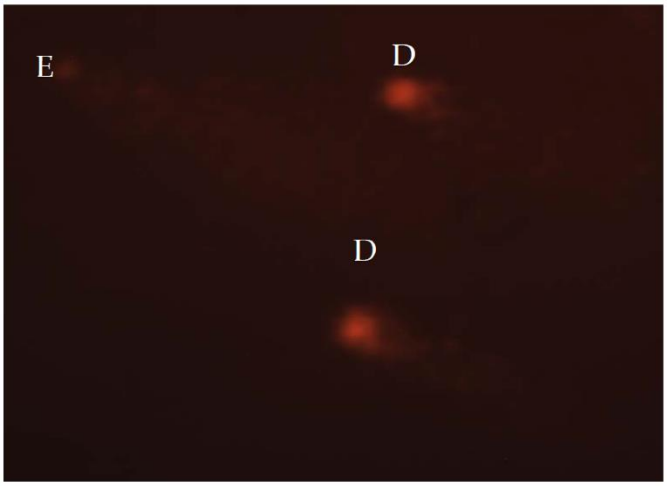

Fig.3. D=high damage nucli, E= total damage nucli, slide is stained with Ethidium bromide seen under flurescent microscope

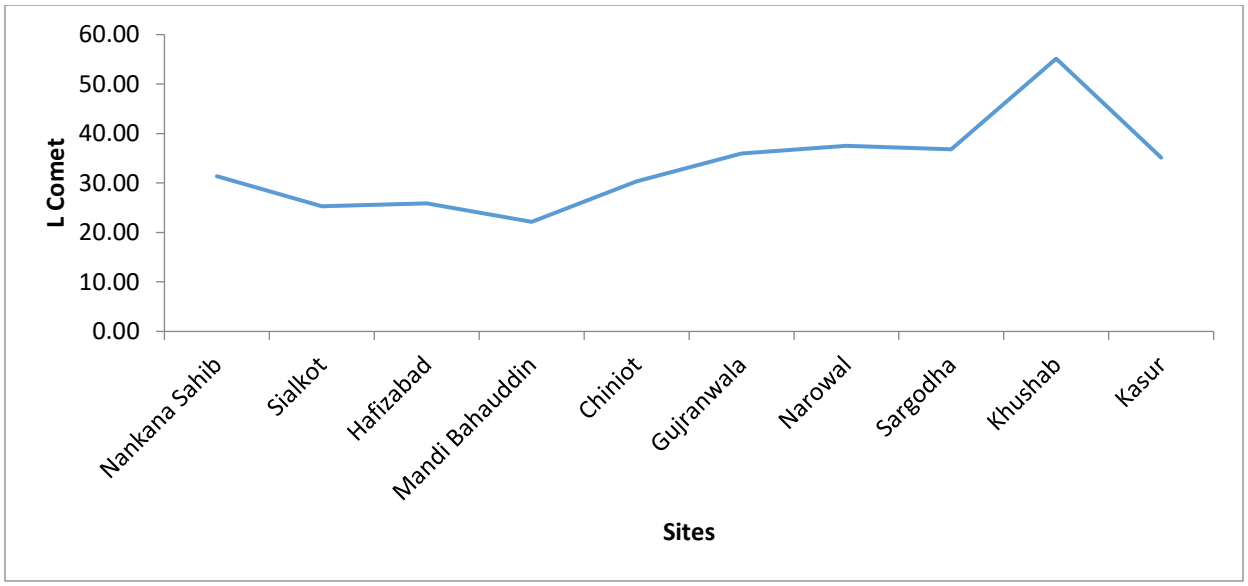

Fig 3. Graphic representation of DNA damage, the length of comet

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Reference

1) Arena, M., & Sgolastra, F. (2014). A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology*, *23*(3), 324-334.
2) Fulton, C. A., Hartz, K. E. H., Fell, R. D., Brewster, C.C., Reeve, J. D., & Lydy, M.\, J., (2019). An assessment of pesticide exposures and land use of honey bees in Virginia. *Chemosphere*, *222*, 489-493.
3) García-Valcárcel, A. I., Martínez-Ferrer, M. T., Campos-Rivela, J. M., & Guil, M. D. H. (2019). Analysis of pesticide residues in honeybee (*Apis mellifera* L.) and in corbicular pollen. Exposure in citrus orchard with an integrated pest management system. *Talanta*, *204* (1), 153-162.
4) Gashout, H.A.; Guzman-Novoa, E.; Goodwin, P.H. (2020). Synthetic and natural acaricides impair hygienic and foraging behaviors of honey bees. *Apidologie*, 51, 1155–1165

5) Glavini´, U., Rajkovi´, M., Ristani´, M., Stevanovi´, J., Djeli´, N., Stanimirovi´, Z., Vejnovi´, B. (2023). Genotoxic Potential of Thymol on Honey Bee DNA in the Comet Assay. *Insects*, 14, 451: 1-12 https://doi.org/10.3390/insects14050451

6) Hayat, K., Afzal, M., Aqueel, M. A., Ali, S., Saeed, M. F., Khan, Q. M., & Damalas, C. A. (2018). Insecticide exposure affects DNA and antioxidant enzymes activity in honey bee species *Apis florea* and *A. dorsata*: Evidence from Punjab, Pakistan. *Science of the Total Environment*, *635*, 1292-1301.

7) Irshad, H., Irfan, S., Noureen, A., Ali, A., Inamullah, Badshah, F., Malook, M.B. (2023). DNA
   a. Damage in Predatory Insects Comet Assay and Different Bioassay for Insecticides in Insects. *Pakistan Journal of Medical & Health Sciences*, Vol 17 (05):652-657
   b. DOI: https://doi.org/10.53350/pjmhs2023175652

8) Magesh, V., Zhu, Z., Tang, T., Chen, S., Li, L., Wang, L., & Wu, Y. (2017). Toxicity of Neonicotinoids to honey bees and detoxification mechanism in honey bees. *International Organization of Scientific Research (IOSR) Journal of Environmental Scientific Toxicology Food Technology*, *11*, 102-110.

9) Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M. T., Tosi, S., Bogo, G., & Bosch, J. (2017). Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Management Science*, *73*(6), 1236-1243

10) Stanimirovic, Z.; Glavinic, U.; Jovanovic, N.M.; Ristanic, M.; Milojkovic-Opsenica, D.; Mutic, J.; Stevanovic, J (2022 a). Preliminary trials on effects of lithium salts on Varroa destructor, honey, and wax matrices. *J. Apic. Res*. 61, 375–391.

11) Stanimirovic, Z.; Glavinic, U.; Ristanic, M.; Jelisic, S.; Vejnovic, B.; Niketic, M.; Stevanovic, J. (2022). Diet supplementation helps honey bee colonies in combat infections by enhancing their hygienic behaviour. *Acta Vet*. 72, 145–166.

12) Stoner, K. A., Cowles, R. S., Nurse, A., & Eitzer, B. D. (2019). Tracking pesticide residues to a plant genus using palynology in pollen trapped from honey bees (Hymenoptera: Apidae) at ornamental plant nurseries. *Environmental entomology*, *48*(2), 351-362.

13) Winfree, R., Williams, N.M., Dushoff, J., Kremen, C (2007). Native bees provide insurance against ongoing honey bee losses. *Ecology Letter*, *10* (11), 1105–1113