VOLUME 9 ISSUE 2 2023

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 9, No. 2, 1275-1287 (2023)

International Journal of Zoological Investigations
Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Insecticidal Efficacy of Copper Oxide Nanoparticles Derived from *Typha australis* Leaf Extract for Controlling *Trogoderma granarium* and Gene Expression Levels after Treatment with Nanobioinsecticide

Pattabhi Ravindra[1] and Subbarao Mannam[2]*

[1]Department of Biochemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Andhra Pradesh, India
[2]Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Andhra Pradesh, India

**Corresponding Author*

Received: 27th September, 2023; **Accepted:** 5th November, 2023; **Published online:** 14th December, 2023

https://doi.org/10.33745/ijzi.2023.v09i02.143

Abstract: Storage insects, particularly the *Trogoderma granarium* (Khapra beetle), reduce the quantity and quality of products that are kept in storage. This species is dangerous because it infests warehouses and makes management more difficult. In addition, containment is required. The preserved grains are home to this insect. Its presence degrades product quality and puts public health in danger. Additionally, it is tough and resistant to a variety of insecticides. In recent years, nanomaterials derived from nanotechnological methods have been proposed as effective insecticide alternatives. Green synthesis was used in this study to investigate the insecticidal properties of *Typha australis* Schum & Thonn leaf extract based copper nanoparticles against *T. granarium*. The extract was used to develop copper nanoparticles (CuO NPs). At 250 and 300 ppm, insecticides killed 90% of *T. Granarium* (*Tg*). In this study, RT-qPCR was performed to analyse *Tg*-SOD, *Tg*-CAT, and *Tg*-GPX gene expression. The results showed that flies fed CuO NPs had much increased levels of expression of these genes. Synthesised CuO NPs may be successful in insect control and a viable component of integrated insect management systems, based on mortality rates and enhanced gene expression levels. More research is thus recommended to properly understand the subject.
Keywords: *Typha australis*, Leaf extract, Green synthesis, CuO NPs, Gene expression, *Trogoderma granarium*, Mortlaity

Citation: Pattabhi Ravindra and Subbarao Mannam: Insecticidal efficacy of copper oxide nanoparticles derived from *Typha australis* leaf extract for controlling *Trogoderma granarium* and gene expression levels after treatment with nanobioinsecticide. Intern. J. Zool. Invest. 9(2): 1275-1287, 2023.

https://doi.org/10.33745/ijzi.2023.v09i02.143

Introduction

In the realm of agriculture, insecticides serve a pivotal function in the control of agricultural pests. However, the emergence and persistence of resistance to these chemical agents has evolved into a significant and persistent global issue (Tudi *et al.*, 2021). Many insecticides, such as melatonin, dichlorvos, Pirimiphos-methyl and fumigant methyl bromide, which are significantly used to control warehouse insects, have been banned (Hamel, 2014). In Asia, populations of Indianmeal moth, Wheat weevil, Red flour beetle, Rice moth, and Khapra beetle have been documented among several insect species that have evolved strong resistance to insecticides (Hamel *et al.*, 2020). The accumulation of high amounts of agrochemical pollutants in soil and water is another problem caused by the use of traditional insecticides. On the other hand, there is a relentless effort to halve the use and risk of chemical insecticides by 2030 (Hamel *et al.*, 2020).

Insects found in warehouses have been observed to result in a substantial reduction in both the quantity and quality of stored products on an annual basis (Shivananjappa *et al.*, 2020). Despite the widespread use of insecticides, these insects continue to destroy stored cereals, especially wheat grains. Damage to stored grains is about 9% in developed countries and 30% in developing countries (Hamel *et al.*, 2020). There are more than 300 different types of stored crop insects, but only 18 of them are of high economic importance.

The Khapra beetle *Trogoderma granarium* (Coleoptera: Dermestidae) is a cereal insect with various physiological features that complicate its control. This insect, which first appeared in India, is considered the most dangerous insect of products stored in many countries in Africa, Asia and Europe. *Trogoderma granarium* (*Tg*) larvae are able to enter diapause in various adverse biotic and abiotic conditions due to temperature, relative humidity, lack of food and high population density (Athanassiou *et al.*, 2019). Diapause is a temporary interruption of the physiological and metabolic state of insects due to various factors. This condition is genetically programmed, and insects that enter diapause are able to respond developmentally to adverse situations through major physiological changes (Wang *et al.*, 2020).

The Khapra beetle*, Trogoderma granarium* (Fig. 1), feeds on a variety of stored cereal grains and is classified among the 100 most harmful invasive insect species (Gourgouta *et al.*, 2021). The Khapra beetle is considered a quarantine insect by the EPPO (European and Mediterranean Plant Protection Organization) (Eppo Global Database, 2021). This insect was first reported in the Indian subcontinent, then spread in hot and arid regions of various regions, including Africa, Europe, the Middle East, Australia and the Americas. For example, India has faced bans from the United States (US) and China due to this quarantine insect (Yadav *et al.*, 2017). Many countries, including the U.S., Canada, and Australia, implement strict quarantine measures to prevent the spread of *T. granarium*. The Committee of the World Trade Organization restricts the import of products contaminated with wheat and other stored grains to prevent the spread of this insect (Athanassiou *et al.*, 2019).

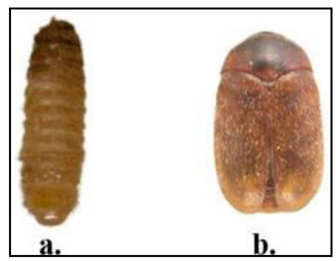


Fig. 1: Khapra beetle larva (a) and adult (b).

The effectiveness and sustainability of using insecticides to control stored crop insects cannot be used efficiently due to the resistance that agricultural and warehouse insects develop to major insecticides. Currently, the use of many insecticides is no longer recommended due to concerns about their harm to human health, ecosystems and non-target organisms. Therefore, alternative insecticides are urgently needed to protect the environment and living organisms. Persistent, excessive or improper application of conventional insecticides, especially fumigants, has led to the development of significant resistance among storage insects. Resistant insect species spread rapidly, causing this situation to become more serious. For example, the indiscriminate and widespread use of malathion, a variety of insecticide, in insect control, is a case of stored product such as *Trogoderma granarium* and *Tribolium castaneum* has led to the fact that insects acquire resistance to this insecticide

(Vivekanandhan *et al.*, 2021). In some regions, phosphine resistance has emerged in various species of stored crop insects, resulting in insect control failures and economic damage. Today, quite common and successfully applied insect control approaches are insufficient in the fight against *T. granarium*.

Recently, the scientific field called "nanotechnology" is expected to offer promising, reliable, and alternative ways to control agricultural and stored crop insects (Periakaruppan *et al.*, 2023). Nanotechnology is the production of nano-sized materials by bottom-up or top-down approaches, and these are called nanoparticles (NP). The bottom-up approach is the aggregation of atoms on a measurement scale of 1 – 100 nm. In the top-down approach, the mass of a substance is reduced to its nanoscale forms (Jayaraj *et al.*, 2019). The synthesized NPs acquire unique and distinct properties that allow for greater usability compared to their previous forms (Worrall *et al.*, 2018).

Nanoparticles in the control of plant and warehouse insects:

Infestation of grain crops by insects is a worldwide problem, causing a loss of between one-quarter and one-half of the annual harvested crop. NPs in many nanoformulation structures have emerged as a promising alternative to control insects. These NPs can be in the form of nanoformulations, nanoemulsions or nanosuspensions. The use of NPs can overcome the limitations associated with conventional insecticides by increasing the effectiveness of insecticides and the stability of active ingredients, reducing the required insecticide dose and preserving agricultural inputs. The use of nanoparticles as insecticides can reduce the toxicity problem caused by overuse of insecticides. Biosensors based on green CuO NPs can be used for insect control and detection of food spoilage diseases. Due to these and many other advantages, it is predicted that nanotechnology may be the best option to improve the current food and agriculture industries (Badawy *et al.*, 2021).

According to research, it has been proven that NPs have higher potential effectiveness in controlling plant diseases and insects. For example, silver, zinc oxide, iron and many other NPs have shown highly efficient biocidal activity against insects (Pittarate *et al.*, 2021). Results from various studies suggest that NPs may be used as an alternative insect control agent against insects in the future (Athanassiou *et al.*, 2018). Metals, metal oxides and other types of NP can be synthesized biologically, physically, and chemically. The synthesized nanometals acquire special, unique and improved physical, chemical and biocidal properties. For example, when the mass of copper solutions such as copper sulfate and copper nitrate is reduced to nanoforms, they acquire highly conductive and biocidal properties. Physical and chemical methods are among the less preferred approaches compared to biological methods due to expensive equipment, hazardous reagents, and the excess energy required to reduce and stabilize NPs (Akintelu *et al.*, 2020).

Green or biological synthesis approaches use living organisms or their components as reducing and stabilizing agents in the synthesis of NPs. This method is affordable, effective and environmentally friendly. Copper nanoparticles (CuO NPs) has been successfully synthesized using various plant extracts with a biological synthesis approach. Biologic agents used for the synthesis of NPs include bacteria, cyanobacteria, fungi, yeasts, algae, and plants (Letchumanan *et al.*, 2021).

Various plant extracts from Typhaceae family have been shown to be efficient, non-toxic and environmentally friendly in the green synthesis of NPs (Uttpal *et al.*, 2022). Although it has been established by bioassay studies that NPs cause insect death, there are limited studies on the underlying mechanisms. In this study, CuO NPs were synthesized using *T. australis* extract with green synthesis approach (CuO NP*)*. Plant extract was used efficiently as a covering agent. Insecticidal effect of CuO NPs bioassay method against *T. granarium* has been studied. The expression levels of three detoxifying genes of

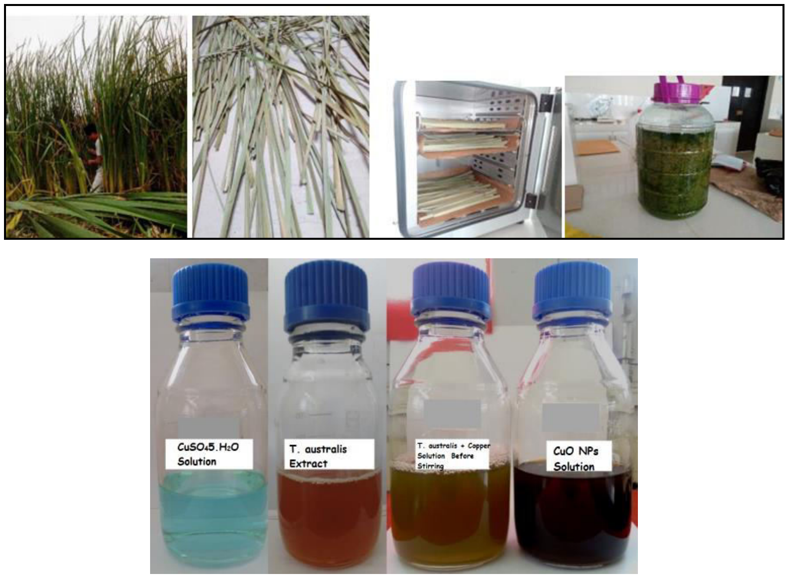


Fig. 2: *Typha australis* leaf and extraction process (above) and CuO NPs development process from the leaf extract and $CuSO_4$ (below).

T. granarium, Superoxide dismutase (*Tg*-SOD*),* Catalase (*Tg*-CAT) and Glutathione Peroxidase (*Tg*-GPX) were analyzed by RT–qPCR to investigate the insecticidal activity of CuO NPs. As a result of bioassay and gene expression analysis studies, the insecticide efficacy of CuO NPs against *T. granarium* has been revealed.

Materials and Methods

Collection and Identification of plant material:

Leaves of *Typha australis* was collected in February 2018from agricultural fields of Guntur, Andhra Pradesh, India. Professor T.N. Mary confirmed the botanical identity of the plant, and a specimen (2211/18) was deposited in the Herbarium of the Taxonomy Laboratory of Acharya Nagarjuna University, India.

Preparation of Extract from Typha australis Leaf:

The fresh leaves of *Typha australis* Schum.& Thonn (Fig. 2) collected were meticulously selected, discarding all those that presented interruptions in the integrity of the leaf blade, insect attacks, scars from blows, etc. The selected material was subjected to drying in the air oven at 102^{o}C for 24 h. Ultimately, the parts were ground in a mechanical mill, yielding 881 g of leaves. The dried and powdered leaves were macerated with hexane (440 g of dry powder in 2 L of solvent) and Ethyl acetate (440 g of dry powder in 1.5 L) at room temperature.

To carry out the green synthesis of CuO NPs, 10 g of dry powdered leaves of *Typha australis* was dissolved in 100 ml of distilled water at a w/v ratio of 1:10. The mixture was heated at 90°C for 15 min. After 15 min, the mixture was filtered using Whatman number 1 filter paper. The samples were centrifuged (Remi Shaker 2L, India) at 9000 rpm to remove plant residues. The resulting supernatant was carefully separated from particulate matter and the clear extract was transferred to a new clean tube. The supernatant was stored at 4^{o}C for subsequent experimental use.

Obtaining Nanoparticles from the extract via Green Synthesis:

10 mM copper sulphate pentahydrate $CuSO_4$ (H_2O) (Sigma – Aldrich) solution was prepared for the green synthesis of CuO NPs. 15 ml extract of *Typha australis* was added dropwise to the prepared copper sulphate solution and the solution was left to stir for 15 min. Then, the pH of the mixture was adjusted to 9 using 1 mM NaOH and the mixture was left to stir for 2 h. After the color change occurred, the NP-containing solution was

centrifuged at 9000 rpm to get rid of agglomerates. Supernatants containing NP were transferred to new sterile tubes. To optimize the CuO NP synthesis conditions, the experiments were repeated by changing the amount of extract used, Cu concentration, reaction time, pH and temperature parameters. To find the optimum amount of extract, different volumes of extract were added dropwise to the copper solution and allowed to mix. To determine the optimum CuSO4 concentration, varying concentrations of $CuSO_4$ (5, 10, 15 and 20 mM) were determined with the optimum extract volume. To determine the optimum reaction time for creating efficient CuO NPs, reactions were carried out starting from 15 min up to 120 min, and spectrophotometric analysis was performed every 15 min. Additionally, syntheses were performed at different pH and temperatures for the optimum pH and temperature of the reaction. For optimum pH determination, the synthesis was carried out at pH: 6, 7, 8, 9, 10 and 11, keeping the reaction time as 75 min. Temperature optimization was performed by setting the reaction temperature to 25 °C, 55 °C and 85 °C. At the end of the reactions, a dark brown color transformation was observed. The first qualitative determination of the synthesis of CuO NPs was made.

Insect breeding:

To grow Khapra beetles, wheat grains were sterilized at 55 °C for 6 h. *Trogoderma granarium* culture was grown in plastic pots (11.5 cm diameter x 18.5 cm height). Tiny holes were made in the lids of the plastic containers and covered with a thin mesh fabric for ventilation. 500 g of wheat and 100 late-stage larvae were added to each plastic container to ensure breeding in the oven at 35 ± 1 °C and 65 ± 5% relative humidity. Larval development periods of the new generation were followed. Pupae were collected to use newly emerged adults for bioassay and gene expression analysis studies.

Bioassay experiment:

Newly emerged adults were collected and CuO NP feeding experiment was performed. For this purpose, 20 g of wheat was weighed and added to 12 different petri dishes. 2 ml CuO NPs at different concentrations of 200, 250 and 300 ppm were distributed homogeneously and carefully into each petri dish. Drying of NPs-treated wheat was carried out using an oven at 35 ± 1 °C for 40 min. 20 *T. granarium* adults that had just emerged from pupae were transferred to petri dishes containing 20 g of wheat treated with CuO NPs and allowed to feed. Mortality of insects was recorded after 12, 24, 48, 72, 96, 120 and 144 h. To perform control experiments, the same steps above were followed, but ddH_2O was used instead of CuO NPs. Each experiment was performed in triplicate and 50% and 90% mortality rates (LC_{50}, LC_{90}) were calculated for each concentration.

Investigation of the effect of CuO NPs on detoxification enzyme genes in Trogoderma granarium:

Expression levels of oxidative stress-sensitive genes, including Superoxide dismutase (*Tg*-SOD), Catalase (*Tg*-CAT) and Glutathione peroxidase (*Tg*-GPX), were examined using the Quantitative Simultaneous PCR (RT - qPCR) technique. This study included total RNA isolation, quantification of isolated RNAs, cDNA synthesis, quantification of synthesized cDNAs, RT - qPCR and statistical analyses.

RNA isolation:

Adults were collected for each experimental set including 12, 24, 48, 72, 96, 120 and 144 h of application for RNA isolation. Total RNA was isolated using NucleoZOL Reagent (Machery-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. Insect samples were placed in a microcentrifuge tube containing 500 µl reagent and homogenized with the help of a micro insectle. After 15 sec of vigorous shaking and 5 min of incubation at room temperature, samples were centrifuged at 12,000 × g for 15 min. Supernatants containing RNA were transferred to new and clean microcentrifuge tubes. RNAs were then precipitated by adding isopropanol. Visible RNA pellets formed at the bottom of the tube were washed twice with 500 µl of 75% ethanol. Finally, the pellets were diluted by adding RNase-free water, and the amount of RNAs and their absorbance at 260 nm and 280 nm were determined using UV-Visible Spectrophotometer (SL 342 – Elico, India).

Complementary DNA (cDNA) synthesis:

RNA isolated from samples was converted to cDNA using the OneScript® Plus cDNA Synthesis Kit (ABM Good, Canada) according to the manufacturer's recommended protocol. For a total reaction volume of 20 µl, the recommended cDNA synthesis components included 4 µl 5X RT buffer, 1 µl dNTP, 1 µl Oligo - dT primer, OneScript® Plus RTase and 1 µg RNA, while the remaining volume was reduced to 20 µl with ddH_2O. completed. The cDNA synthesis reaction consisted of incubation for 15 min at 55 °C and 5 min at 85 °C. At the end of the incubation, the samples were quickly cooled on ice and the cDNAs were stored at -20 °C for subsequent applications.

Designing suitable primers for Trogoderma granarium detoxification genes:

Four unigenes encoding *Tg*-SOD, *Tg*-CAT, *Tg*-GPX and the housekeeping gene ß-actin (TgACT) were found from the previously created cDNA library of *T. granarium* (Dağeri, 2022). Primers were designed using the Integrated DNA Technologies (IDT) (https://eu.idtdna.com/Primerquest/ Home/ Index) site.

Primers used in the study:

Sequence of Gene Primers (5'-3')

- ***Tg*-SOD:** F: CACTGGTAAGCGGCAGTCGT; R: CGCTCCCAGACTCGATTGCA
- ***Tg*-CAT :** F: GACCTCTCTGGGATAGACCC; R: GTGGCGCACCACTTTCATTG
- ***Tg*-GPX :** F: GCTGATCGATGGCGTCTTCA; R: CCTTAGATCAGCACGCGAGTT
- **TgACT :** F: AGGAGCGCGCAGGTAAATGTTG; R: CCACCTATTTAGGCAACATCC

Quantitative Simultaneous PCR (RT-qPCR):

RT-qPCR experiments were performed using QuantStudio 3 Real-Time PCR System (Applied Biosystems) using 50 ng of cDNA to examine the transcriptional regulation of CuO NP-treated samples expected to respond to oxidative stress. Reactions were performed using FastStart Essential DNA Green Master (Roche) in a total reaction volume of 20 µl. RT - qPCR conditions included a pre-incubation at 95 °C for 10 min, followed by 10 sec at 95 °C, 10 sec at the specific binding temperature of the primers, 40 cycles at 72 °C for 10 sec, followed by 40 cycles at 40 °C. It consisted of cooling steps for 10 sec. Following each reaction, a melting curve analysis was performed to verify the integrity of each fragment and control for contamination according to the manufacturer's instructions (Table 1).

Each reaction was carried out in three technical replicates. The relative quantitative method $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of *Tg*-SOD, *Tg*-CAT, and *Tg*-GPX (Livak and Schmittgen, 2001).

Statistical analysis:

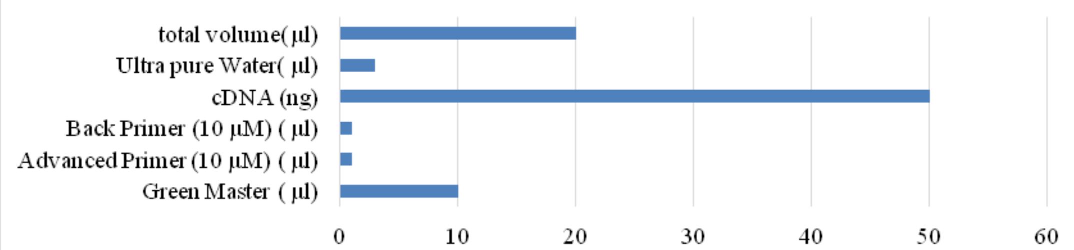
Statistical evaluation was performed using Minitab 19.0 (Minitab Ltd, Brandon Court, United Kingdom) software. Determination of statistical significance differences between applications was made using one-way analysis of variance (ANOVA) followed by the Tukey HSD (Honestly Significant Difference) test. p value of less than 0.05 was considered statistically significant.

Results

Synthesis of Copperoxide Nanoparticles:

In the synthesis of copper nanoparticles, the reaction mechanism involves reduction of copper ions to copper atoms, nucleation of copper nanoparticles, and subsequent nuclear growth while the copper nanoparticles are coated with extract components. This is due to the fact that extract components often act as reducing and coating agents in the synthesis of nanoparticles (Pandit *et al.*, 2022). The first indicator of nanoparticle synthesis is color change (Fig. 3), so *T. australis* in the synthesis of CuO NPs. The

Table 1: RT - components used for qPCR study



	Green Master (µl)	Advanced Primer (10 µM) (µl)	Back Primer (10 µM) (µl)	cDNA (ng)	Ultra pure Water(µl)	total volume(µl)
Volume	10	1	1	50	3	20

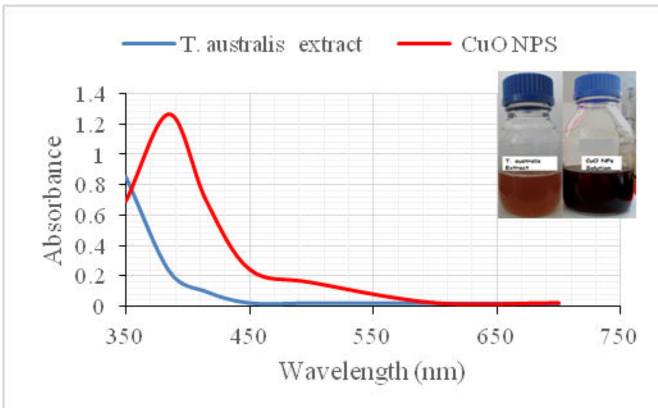


Fig. 3: CuO NPs (blue) and *T. australis* UV/Visible absorption spectra of the extract (red).

effectiveness of the plant as a bioreductant and biosealant was determined by monitoring the color change of the reaction mixture.

Two simultaneous experiments were conducted to investigate the effectiveness of the plant *T. australis* as a bioreducing and biocoating agent in the synthesis of CuO NPs. In the first experiment, 10 mM $CuSO_4$ solution was prepared by dissolving 0.156 g of $CuSO_4$ $5H_2O$ crystals in 100 ml of distilled water. 15 ml of copper solution was added dropwise to a 100 ml beaker on a magnetic stirrer and 15 ml of extract was added to the mixed solution. The reaction was continued at room temperature without pH adjustment for 2 h and overnight if there was no color change. The approximate pH of the reaction mixture was found to be 5. In the second experiment, 15 ml of extract was added dropwise to 15 ml of 10 mM $CuSO_4$ solution and mixed for 15 min. Then, the pH of the reaction mixture was adjusted to 9 and stirred at room temperature for 2 h. While there was a very slight color change during overnight stirring in the first experiment, the light brown color of the reaction mixture at the beginning of the reaction turned to dark brown after adjusting to pH: 9 in the second experiment (Fig. 2).

As a result of the synthesis without pH regulation suggests that the role of the extract is only to stabilize copper ions, not to reduce them. Because, there was no significant color change for the formation of copper nanoparticles in the synthesis. This result indicates that *it*may be due to the low capacity of the extract to reduce Cu^{2+} ion to Cu0 atom, because the strength of the reduction potential is not sufficient to reduce copper ions to copper atoms at pH: 5. To confirm the formation of CuO NPs and identify SPR peaks, a UV - Visible absorption spectrum was taken for the sample synthesized by pH regulation (Fig. 3).

In Figure 3, UV / Visible absorbance peaks of CuO NPs at 385 nm is the YPR band of CuO NPs synthesized with the extract. Because the GPR bands of nano-sized particles change and differ in the use of various coating agents.

Feeding of Khapra Beetle Bioassay with CuO NP:

In past studies, Ag NPs (Almadiy *et al.*, 2018), silicon oxide (SNPs), aluminum oxide (ANPs) and zinc oxide (ZNPs), silica NPs, AEROSIL 200 were applied against *T. granarium*, where CuO NPs have not been applied so far (Akintelu and Folorunso, 2020; Badawy *et al.*, 2021). The insecticidal effect developed in *T. granarium* adults fed with wheat grains supplemented with CuO NPs at concentrations of 200, 250 and 300 ppm was observed every 12, 24, 48, 72, 96, 120 and 144 h. It was observed that as NP concentration and exposure time increased, the mortality rate of insects also increased significantly. LC_{50} value was determined 72 h after 250 and 300 ppm concentration applications. The LC_{90} value was found after 144 h of treatments at both 250 and 300 ppm concentration. The LC_{50} value of the 200 ppm concentration was determined at the end of the 144th h. No mortality was observed in control samples until the 120th h. Thus, it is obvious that concentrations at both 250 and 300 ppm cause death in adult insects with a LC_{50} mortality rate at 72 h. Additionally, both 250 ppm and 300 ppm concentrations provided a similar insecticidal effect after the 120th h. According to the bioassay results, applications of CuO NPs at different concentrations showed insecticidal agent properties against *T. granarium* (Fig. 4).

Expression level analysis of Tg-SOD, Tg-CAT and Tg-GPX genes after CuO NP treatment:

The highest amount of *Tg*-SOD transcripts were detected after the 48th and 144th h, respectively, of feeding Khapra beetles with wheat grains treated with CuO NPs at a concentration of 200 ppm. *Tg*-SOD level was downregulated after 12th, 24th, 72nd, 96th and 120th h of 200 ppm concentration treatment. The highest amount of *Tg*-SOD transcript was detected in *T. granarium* adults after the 144th and 72nd h of feeding with wheat grains treated with CuO NPs at a concentration of 300 ppm. The gene expression level was found to be almost insensitive after the 48th and 96th h of 300 ppm application. Additionally, the mRNA level of *Tg*-SOD was downregulated after the 12th, 24th and 120th h of 300 ppm concentration application (Fig. 5).

Tg-CAT expression level was examined at different time points (12, 24, 48, 72, 96, 120 and 144 h) on newly hatched *T. granarium* adults fed with wheat grains treated with 200 and 300 ppm CuO NPs. The results showed that the highest (8.4-fold) *Tg*-CAT expression was detected after 48 h of feeding with CuO NP-treated wheat grains at a concentration of 200 ppm. *Tg*-CAT expression was also upregulated after 96 h of feeding at concentrations of 200 and 300 ppm. Slightly upward *Tg*-CAT expression levels were observed after 144 h of treatment for both concentrations. Gene levels monitored following the 24th and 120th h of both applications, the 72nd h of 200 ppm and the 48th h of 300 ppm concentration were found to be almost insensitive. On the other hand, *Tg*-CAT was downregulated after the 12th h of 200 and 300 ppm concentration applications and after the 72nd h of 300 ppm application (Fig. 6).

The highest amount of *Tg*-GPX transcript was observed after the 48th, 72nd and 96th hours of feeding with wheat grains treated with CuO NPs at a concentration of 200 ppm, respectively.

Tg-GPX expression, 200 ppm concentration treatment:

It was found to be virtually insensitive after 144 h. The expression level of *Tg*-GPX was down-regulated after 12th, 24th, 120th h of 200 ppm application. *Tg*-GPX was expressed at the highest level after the 72nd, 96th and 24th h of feeding with wheat grains treated with CuO NPs at a concentration of 300 ppm, respectively. It was

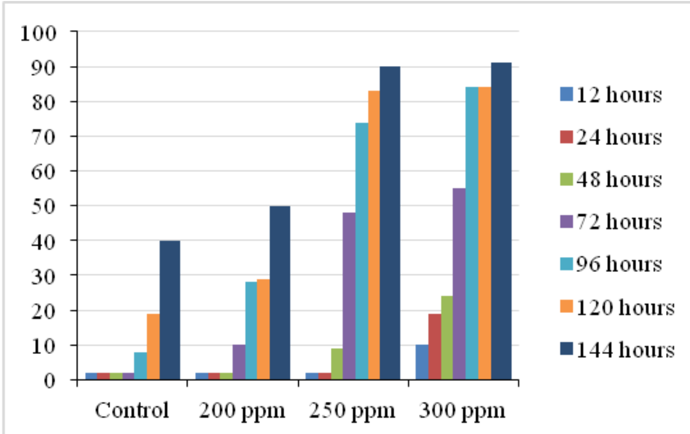


Fig. 4: Mortality rates obtained after feeding Khapra beetle with CuO NPs.

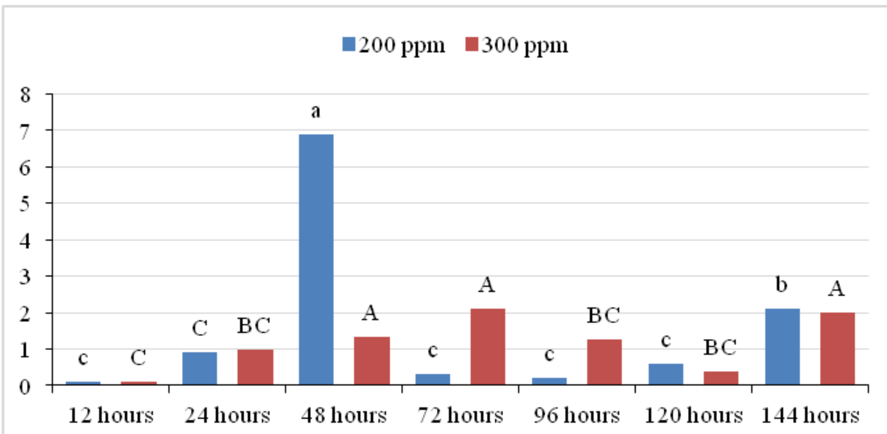


Fig. 5: *Tg*-SOD gene expression levels after CuO NP treatment.

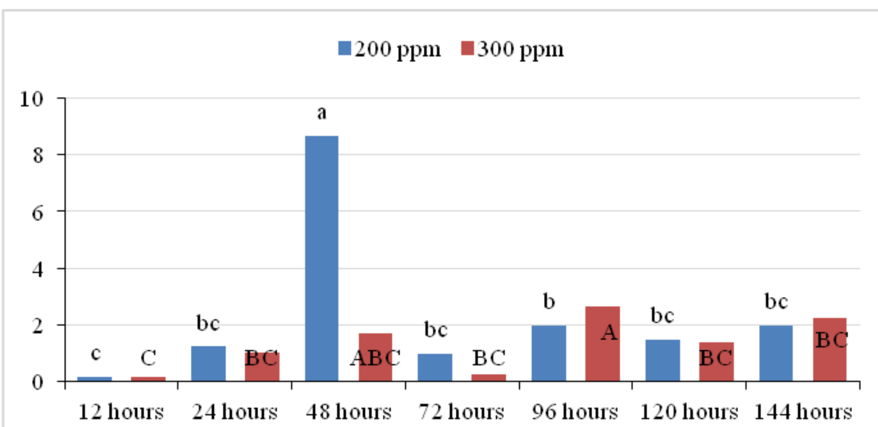


Fig. 6: *Tg*-CAT gene expression levels after CuO NP treatment.

observed that the expression level of *Tg*-GPX was almost insensitive after the 48th, 120th and 144th h of 300 ppm application. Moreover, it was downregulated after the 12th h of 300 ppm concentration application (Fig. 7).

Discussion

The larvicidal activity of green synthesized CuO - NPs using the leaf extract of *Tridax procumbens* on the dengue, Zika and chikungunya disease vector *Aedes aegypti* was investigated by Selvan *et al.*

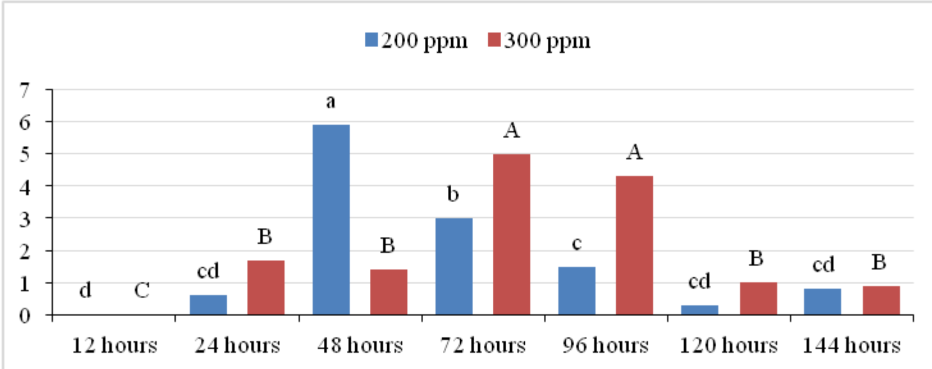


Fig. 7: *Tg*-GPX gene expression levels after CuO NP treatment.
The values with different superscript letters (a, b, c/A, B, C) in a data label of Figures 5, 6 and 7 are significantly different ($p<0.05$). Each superscript letter that is different for each parameter shows a significant difference. The superscripts mean that a/A and b/B are not significant from each other in a statistical sense. Also, that c/C is not significantly different. However, significant differences were found between A and B, between B and C, and between b and c (among other differences).

(2018). Researchers have reported a strong larvicidal effect of CuO NPs with a high mortality rate. As a result of the application of CuO NP biosynthesis from wet leaves of *Artocarpus heterophyllu* against the first and fourth instar larvae of *Aedes aegypti*, highly insecticidal effects were detected. Within 12 h of exposure to synthesized CuO NPs mediated by Sambong (*Blumea balsamifera*) extract, mortality rates ranging from 25 to 100% have been detected in fruit flies (Almadiy *et al.*, 2018). The insecticidal activity of CuO NPs synthesized from *Annona squamosa* L. seed against *Anopheles stephensi*, *Tenebrio molitor* and non-target *Artemia nauplii* was reported by Vivekanandhan *et al.* (2021). It was concluded that CuO NPs had a significant effect in controlling insect larvae and reducing toxicity towards non-target *Artemia nauplii*. It was concluded that using CuO NPs obtained from *A. squamosa L.* seed in insect control may be a faster, safer and greener option. 20 ppm CuO NPs synthesized with *Lantana camara* extract caused 100% larval death in *Anopheles mosquitoes*. However, the same effect was observed after using the plant extract alone at 140 ppm (Kamel, 2022). Two-spotted spider mite *Tetranychus urticae* (TTSM) Koch is one of the most important agricultural insects of fields, greenhouses, vegetables and fruits. Dorri *et al.* (2018) tried to control TTSM by spraying copper nanocapsule (Cu_2O) at 5 different concentrations. They reported that the TTSM population decreased significantly after the application of CuO nano-capsule.

Biosynthesis of different types of NPs across a wide range of potential sites is not limited to plants alone. In a past study, El-Saadony *et al.* (2020) biosynthesized Cu-NPs with sizes ranging from 10 to 70 nm using the *Pseudomonas fluorescens* strain and revealed that these Cu-NPs have a significant insecticidal effect with very low concentration. The LC_{50} value was found to be 37 ppm after 5 days of treatment against the stored product insect *T. castaneum*. It was also concluded that these biosynthesized Cu-NPs are more effective than chemically synthesized Cu-NPs. Entomopathogenic *Metarhizium robertsii*-based biosynthesized, 15.67-62.56 nm sized, CuO NPs showed significant insecticidal activities against insects including *Anopheles stephassi, Aedes aegypti, Culex quinquefasciatus* and *Tenebrio molitors*. LC_{90} concentrations were found to be doses of 23, 717 µg/ml, 65,144 µg/ml, 14,997 µg/ml, and 29,363 µg/ml, respectively (Vivekanandhan *et al.*, 2021).

In this study, CuO NPs synthesized from *T. australis* were applied for the first time to investigate their insecticidal effect on *T. granarium* species. The application of green NPs in the fight against warehouse insects and against insects in plant protection areas is a relatively new and developing field. The insecticidal properties of NPs arise from their morphological properties that cause physiological changes. Metals cause toxic effects on the organism by being involved in biochemical reactions that they are not involved in under normal conditions. As a result of this study, it can be concluded that the mortality rates at the concentrations where NPs are applied vary from species to species and NP synthesis method in insects. According to the previous studies performed and the results obtained within the scope of this study, the idea emerges that NPs may be a promising biocontrol agent for insect control and subsequent agricultural improvement studies.

Antioxidant genes have very important roles in the development of resistance among insects due to the elimination of ROS substances. To overcome toxicity, organisms are equipped with an antioxidant protection system consisting of free radical scavengers and antioxidant enzymes to detoxify and eliminate ROS caused by toxic substances. Oxidative biomarkers SOD, CAT, GPX, GST, and AChE were examined in response to Cu accumulation in the midgut and fat body of the model organism *Galleria mellonella*. As a result, it has been shown that antioxidant enzymes are strongly affected by the poisoning of insects with heavy metals (Sezer *et al.*, 2019). The vinegar fly (*Drosophila melanogaster*), which abundantly expresses the CAT gene, has an enhanced resistance to H_2O_2. In contrast, nits flies lacking CAT activity have high levels of H_2O_2 and shorter viability and lifespans. Similar results were found for the mosquito *Culex pipiens*; It has been reported that when CAT expression is inhibited via RNA interference, the survival rate of the adult mosquito is significantly reduced. When dwarf chichlid fish were exposed to CuO NPs, they showed increased CAT activity (Ahmed, 2021).

The upregulated expression of *Tg*-GPX at different hours of insect exposure to CuO NPs indicates that this gene is highly affected by CuO NPs. Additionally, it has been shown in various studies that metal NPs have a significant effect on GPX expression in different organisms. For example, exposure of *Chironomus riparius* to cadmium increased the expression of phospholipid hydroperoxide glutathione peroxidase (CrPHGPx1). This result suggested that CrPHGPx1 is differentially regulated in redox balance. The high *Tg*-GPX expression levels obtained in this study clearly demonstrate the toxic effect of CuO NPs on *T. granarium*. Increased biological reactions to oxidative stress may include DNA damage and fragmentation, cell death, lipid peroxidation, and lysosomal membrane instability. These biological reactions can be used to investigate the harmful effects of NPs (Ahmed, 2021). Altered expression of these enzyme-encoding genes are indicators of stress caused by NPs and indicate the potential toxicity they may cause.

Increased *Tg*-CAT, *Tg*-GPX and *Tg*-SOD gene expression levels in *T. granarium* adults fed with wheat grains treated with CuO NPs indicate that these adults are exposed to oxidative stress and develop an antioxidant response. For example, a past study revealed that CuO NPs showed lethal properties by increasing intracellular ROS formation (Ruiz *et al*., 2015). In this study, it can be concluded that CuO NPs significantly upregulate *Tg*-SOD, *Tg*-CAT and *Tg*-GPX antioxidant genes, which may be related to the potential insecticidal activities of CuO NPs. Moreover, it can be suggested that the high expression of antioxidant enzymes encoding genes is directly related to the ROS products caused by NPs.

Conclusion

This study on the insecticidal effectiveness of the synthesized NPs against the Khapra beetle showed that CuO NPs caused 50% and 90% death of the insect at concentrations of 200 and 300 ppm after 72 and 144 h of application, respectively, and therefore these NPs are effective against the Khapra beetle. It puts forward the idea that it can be used as a potential and alternative insect control agent for insects. The abundant expression of *Tg*-CAT, *Tg*-GPX and *Tg*-SOD genes at various times after the application of CuO NPs indicated that the NPs were responsible for ROS stress in the insect and the toxic effect of CuO NPs on the Khapra beetle. Based on these findings, considering the significant mortality rates caused by CuO NPs on the insect, it can be suggested that these NPs may be effective in the management of the insect and become a potential essential component of integrated insect management (IPM). However, due to the limited studies on the effects of CuO NP and other NP types on Khapra and other warehouse insects, it may be recommended to conduct further research and functional studies on this subject.

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