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Novel and conventional techniques for detecting clonazepam in *Lucilia cuprina* (Diptera: Calliphoridae)

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ABSTRACT

Benzodiazepine drugs, particularly clonazepam, are used as adjunctive therapy to manage epilepsy and control seizures. However, the misuse of these drugs can be fatal. One requirement in forensic investigations is to find evidence of drug residue within a deceased individual's body. In such investigations, entomology can assist by conducting specific pathological and toxicological tests, even when the body is highly decomposed. This study evaluated the effectiveness of two new methods, namely the measurement of protein carbonyls and the use of α,α-diphenyl-β-picrylhydrazyl (DPPH), in detecting clonazepam in *Lucilia cuprina* (Wiedemann, 1830). The insect's larvae were fed on the muscle tissues of clonazepam-injected rabbits. These methods were compared to the conventional analytical technique of "High-performance liquid chromatography-mass spectrometry" (HPLC-MS). Additionally, the impact of the drug on *L. cuprina* development was assessed by measuring morphometric data at the larval, prepupal, and pupal stages. The results demonstrated a significant increase in morphological characteristics (weight, length, and width) in all the groups treated with the drug, when compared to negative and/or positive control groups. Furthermore, the drug caused a noticeable rise in protein carbonyl levels, indicating oxidative damage in the treated groups compared to the control groups. Clonazepam also substantially increased the DPPH inhibition percentage when compared to the negative control. HPLC-MS successfully detected a significant elevation in clonazepam concentration in all the treated *L. cuprina* specimens. Furthermore, the empty pupariam of *L. cuprina* has proven its validity to detect the drug.

Keywords: Forensic Entomology; Entomotoxicology; Benzodiazepines; Oxidative stress; developmental data

INTRODUCTION

The investigation of an inexplicable murder can benefit from the integration of versatile investigative tools, including forensic entomology which proved its effectiveness, especially in the severely decomposed or mutilated carcasses (Pounder, 1991; Waghmare *et al*., 2015; Byrd and Tomberlin, 2019; Bhardwaj *et al*., 2020). Insects are the most constant and diverse arthropod group on earth and several species can be found around the cadavers (El-Bassiony, 2020). Calliphoridae, especially *L. cuprina*, earned the title of "initial colonizers" as they were capable of reaching carrions after seconds, or a few minutes following death (O'Flynn, 1983; Bansode, 2016; Bhardwaj *et al*., 2020). Their sizes and developmental stages could help in the estimated post-mortem interval (Bala and Sharma, 2016), and their empty puparia were stable and endured for a long time (Bourel *et al*., 2001). Furthermore, calliphorid flies offered more sensitive results in comparison

to human specimens in some of the previous entomo-toxicological investigations (Gosselin *et al*., 2010; Groth *et al*., 2022). Xenobiotics could affect the human body's decaying rate as well as the rate of insect aggression and their growth (Campobasso *et al*., 2004; Boulkenafet *et al*., 2020). These hypotheses prompted entomologists to conduct further research studies regarding the effect of xenobiotics on the growth rate and biochemical parameters of forensically important insects. Several dipteran flies have proven their efficacy in detecting Cocaine, amphetamine (Campobasso *et al*., 2001), diazepam (Carvalho *et al*., 2001), flunitrazepam (Barbosa *et al*., 2018), and benzodiazepines (Groth *et al*., 2022). The shortage of supporting references, in addition to the absence of standard measuring methods, hindered the use of forensic entomo-toxicology in criminal or non-criminal death investigations (Hodecek, 2020; Groth *et al*., 2022).

Benzodiazepines, such as clonazepam, are the most utilized drugs to cure panic attacks. They were gamma-aminobutyric acid (GABA) modulator complexes, which influence the GABA receptor-ionophore complex, and therefore were utilized in the treatment of anxiety (Miziak and Czuczwar, 2020). Yet, some studies reported mortality cases as a result of drug misuse (Belleville, 2010). The latter studies were supported by statistics of the National Institute on Drug Abuse, which reported that the victim percentages increased around 4.3-fold in 2015 than in 2002 (Purvez, 2018). Many drugs are capable of being magnified through the food chain and their effects could be analyzed inside the insect tissues (Tabor *et al*., 2005) and through the insect life cycle as well. Due to previous research, the effect of a drug on insects varies according to the insect species (Kharbouche *et al*., 2008; Afifi *et al*., 2022 a&b).

Several techniques have been used to detect the impact of drugs/toxins on forensically important insects. They range from developmental analyses, which involve measuring the weight, length, and width of immatures, and studying insect life cycles (Tullis and Goff, 1987), to quantitative analyses by using HPLC-MS and other analytical methods (Wood *et al*., 2003). Additionally, a color-based technique was used to detect diazepam and other drugs in the blowfly *Calliphora sp*. This method involved detecting the drug from the adult stage in the first generation to the adults in the next one, confirming the insect's capability to serve as forensic samples in death scenes (Gola and Lukose, 2007). However, no research has focused on measuring the damage attributable to the bioaccumulation of essential molecules by employing protein carbonyls amounts and α,α-diphenyl-β-picrylhydrazyl (DPPH) analyses in insects that have fed on poisoned/drugged carcasses. These techniques are low-cost, simple, sensitive, and selective for measuring oxidative stress (Wood *et al*., 2003) and can be practically applied in the field of forensic entomology.

The present work aimed to measure the developmental data (weight, length, and width) of *Lucilia cuprina* immatures fed on rabbits that were previously injected with different clonazepam concentrations. Additionally, the impact of the drug was evaluated on the treated insects by using HPLC-MS, protein carbonyls amount, and DPPH analyses.

MATERIALS AND METHODS

Ethical statement

The current animal-related experiments were performed with the approval of Cairo University Institutional Animal Care and Use Committee (CU-IACUC). All animal experiments were carried out in compliance with Cairo

University Guidelines, regarding CU-IACUC under the number CU09092023752.

Stock colony of flies

Lucilia cuprina was collected on fresh minced buffalo meat from the gardens of Cairo University, and was identified and reared in the Medical Entomology Lab, Entomology Department, Faculty of Science, Cairo University, Egypt, for three generations before using them in this experiment. The adults were provided with water and granulated sugar regularly in 45x45x45 wooden cages. The females were provided with buffalo meat to both develop eggs and oviposit. The hatched larvae were reared on buffalo meat as well. Both the rearing process and the experiments were maintained under laboratory conditions (14L: 10D h cycle at 31.5± 4°C and 52.8%± 5% RH).

Application of clonazepam on experimental rabbits and Insect's treatments

Clonazepam (C1277) Sigma-Aldrich was dissolved in purified distilled water at four concentrations: 0, 0.7, 1 and 6 mg/mL, which represented positive control, normal, toxic, and lethal doses, respectively, according to the Drug Bank (https://go.drugbank.com/). Four Netherland rabbits, *Oryctolagus cuniculus domesticus* (1.00 ± 0.2 kg in weight) were the experimental animals, each was intravenously injected through the curricular vein with the respective drug concentration of clonazepam 1.5 mL, and the animals were left for 6 hrs post-injection to enable drug circulation inside their tissues. A fifth rabbit served as the negative control and was kept for 6 hours with no injections. The rabbits were euthanized by decapitation (Close *et al.*, 1997; Boyal *et al.*, 2022). The rabbits' muscle tissues were used in this experiment as the insects' comestibles. An egg batch (~140 eggs) of *L. cuprina* (El-Bassiony, 2020) was placed on the rabbits' muscle tissues (100 g), and the hatched larvae were allowed to reach the pupal stage, in a 100 mL plastic container covered with a fabric mesh. The control and treated pupae were placed in cages to allow the maturation into adults to take place, and then the empty puparia were collected. Each control or treated group was replicated three times.

Morphometric analyses

Twenty immatures (first, second, third, prepupa, and pupae) were collected regularly, from each control and treated group, identified, killed by immersing them in hot water (90°C) for 10 seconds, dried, and morphologically measured by using a digital caliper (MITYTOUO digital Vernier) and a digital balance (RADWAG, WTB200). Both the immatures and the empty puparia were stored at -20°C until needed in the biochemical and analytical analyses.

Biochemical analyses

The concentration of clonazepam in insects was determined by HPLC-MS analysis (Rojas *et al.*, 2017). Each experimental sample underwent preparation by using an ethanol extraction system. Cartridge C-18 was hardened with 2 ml of methanol and 2 ml of distilled water. The elution of the analyte was obtained using 1ml of 1:1 methanol/distilled water mixture, which was then added to a chromatography vial. Subsequently, 20 µl of the extracted sample was injected into an HPLC-UVDAD Agilent Technologies 1100 series®, equipped with RP-18 end-capped column (5µm), 150mm-4.6 (Purospher® STAR). The mobile phase consisted of 40% v/v acetonitrile and 60% v/v Buffer K2HPO4 / KH2PO4, adjusted to pH 8.5 with triethylamine. The flow rate of this mobile

phase was set at 1 mL/min, and the analysis ran at a wavelength of 245 nm for 11 minutes.

The determination of the protein oxidative damage, in the form of protein carbonyls, was carried out following the Levine *et al.* (1990) method, with minor modifications. Briefly, experimental samples were homogenized in ice-cold phosphate buffer (pH 7.0) and then centrifuged at 2000 ×g for 10 minutes at 4°C. Subsequently, 800 µL of supernatant from each experimental sample was mixed with 200 µL of 2,4-dinitrophenyl hydrazine (DNPH) and incubated for 30 minutes at room temperature. The mixture was then precipitated with 10% trichloroacetic acid (TCA) and left for 10 minutes at 4°C. After centrifugation at 5000 ×g for 7 minutes at 4°C, the pellet was washed four times with an ethanol/ethyl acetate (1:1) mixture and re-dissolved in 1 ml of sodium phosphate buffer (pH 6.8). Finally, the absorbance was measured at 366 nm, and the concentration of protein carbonyls was expressed as OD/mg protein. The total protein concentration of the samples was determined spectrophotometrically following the method of Bradford (1976).

The α,α-diphenyl-β-picrylhydrazyl (DPPH) method, used to detect the non-enzymatic antioxidant response to stress factors (Blois, 1958), relies on measuring scavenging capability. In this assay, DPPH (0.5M) was added to different sample concentrations and incubated for varying times before measuring absorbance at 525nm.

Statistical analysis

Non-parametric analysis was performed, on all the experimental groups, using the Kruskal-Wallis revealing test, with *p*–value (<0.05). All the statistical analyses were performed using IBM SPSS Statistics for Windows (Version 17.0. Armonk, NY: IBM Corp.).

RESULTS AND DISCUSSION

The non-parametric analysis, using the Kruskal-Wallis test, clarified significant ($p < 0.05$) differences in most of the morphological data of the treated larval, prepupal, and pupal stages of *L. cuprina*, in comparison with the two control groups ($\varkappa^2$ = 9.9, 13.5, 13.5; *df* =4, 4, 4; and *p value*<*0.05*, <0.05, <0.05, respectively). The three concentrations of clonazepam (0.07, 1, and 6 mg/mL) elevated the weight (mg), length (mm), and width (mm) significantly in the first, second, and third larval instars, prepupa, and pupa (Fig. 1A, 1B & 1C). The one exception was the pupal length of 0.07 mg/mL in the treated group, which showed significant ($p < 0.05$) and insignificant ($p > 0.05$) differences compared to the negative and the positive controls, respectively. In accordance with our finding, the larvae of *Chrysomya albiceps* (Wiedemann, 1819), and *Chrysomya putoria* (Wiedemann, 1830), fed on diazepam mixed tissues, revealed a significant increase in the developmental rate, with respect to the control larvae (Carvalho *et al*., 2001). In addition to this, the body length and weight of *Lucilia sericata* (Meigen) differed significantly after being treated with different doses of ketamine drug (Zou *et al*., 2013). Also, a direct correlation between the weight parameters of *C. albiceps*, and *C. putoria* larvae, and the diazepam drug concentration was recorded (Carvalho *et al*., 2001). The same responses were recorded for different flies fed on ethanol (Tabor *et al*., 2005), anticholinergic (Oliveira *et al*., 2009), cocaine (de Carvalho *et al*., 2012), and methamphetamine (Goff *et al*., 1997). On the contrary, diazepam slowed down the larval growth in *C. albiceps (*Elshehaby *et al., 2019)*, and also in *L. cuprina* treated with higher concentrations of the drug (Pawar, 2021). In agreement with the current findings, clonazepam affected the morphological measurements of the

developmental stages of *Sarcophaga argyrostoma* (Robineau-Desvoidy, 1830) fed on different concentrations of clonazepam (25, 50, and 100 mg/ml) as an in vitro application, where the higher two concentrations increased the weight, length, and width significantly in the late third instar and prepupa (Afifi *et al*., 2022a).

The detection of drugs in insects was not only helping in the medico-legal forensic application but also in environmental biomonitoring especially when insects were used as biomarker agents (Close *et al*., 1997). Previous data proved the ability of HPLC-MS to detect several drugs in insects. Morphine was analyzed by HPLC-MS in *C. albiceps*, and the drug could be detected in the feeding and post-feeding larvae (Salimi *et al*., 2018). Recently, the effect of Viagra overdose plus diazepam on the third larval stage, pupae, and adults of *C. albiceps* were tested, by using HPLC-MS, and the results ensured the presence of the two tested drugs in all of the insect's developmental instar (Elshehaby *et al*., 2019). In the current research, clonazepam was analytically measured (mg/mL) by HPLC-MS, in the different treated groups of *L. cuprina* (Fig. 2), and its concentration recorded significant increases in all the treated groups ($\varkappa^2$ =11.76, 13.2, 13.7*; df*= 4; *p-value <0.05*) compared to the controls. The fading of the drug level from one instar to the next might imply that the drug converted into its metabolite form, 7-acetamido-clonazepam. At the beginning of the second instar, the drug concentration exhibited its highest level when the larvae fed on the lowest drug concentration (0.7 mg/mL). This substantiates the drug's bioaccumulation in the insect's tissues (Kharbouche *et al*., 2008). This bioaccumulation was inversely proportional to the concentration of the drug in most of the treated groups (Fig. 2). The bioaccumulation of drugs in necrophagous insects was influenced by several factors such as the insect's developmental stage, its feeding area, and/or the physiochemical stability of a drug in this insect (Dalle-Donne *et al*., 2003; Kharbouche *et al*., 2008).

The protein carbonyls analysis was considered an indicator of the oxidative stress factors (Dalle-Donne *et al*., 2003), and it has been used to detect the pesticides in freshwater fish *Channa punctata* (Bloch) (Parvez, and Raisuddin, 2005), the plant phenolic compounds (Renault *et al*., 2018), and even the normal levels of environmental pollutants in insects (Abdelfattah, 2020). This analysis was recommended as an effective method due to the relative stability of protein carbonyls in contrast to other oxidative compounds, besides its low price and simple applicability (Dalle-Donne *et al*., 2003). The current study showed that the damaged protein, in form of protein carbonyls of *L. cuprina*, was elevated in most treated groups and developmental stages with respect to the controls ($\varkappa^2$= 13.4, 12.8; *df*= 4; *p-value <0.05*, respectively) (Fig. 3). In the same context, clonazepam was linked to Alzheimer's disease (AD) and the latter was characterized by a high level of protein carbonyls amount (Ginsburg, 1991). The current data showed a gradual fading of the protein carbonyls amount from one stage to the next, especially in the pupal stage, and this may be attributed to the formation of the clonazepam metabolites, or as a feature related to the dormant pupal stage. Yet, the amount of protein carbonyls declined significantly in all the treated instars, after feeding them on muscle tissues containing the highest concentration of the drug (6 mg/mL), in comparison with the other treated groups (0.7 and 1 mg/mL). This could indicate enzymatic and non-enzymatic responses to the drug or a fluctuation in the balance between protein oxidation and the redox regulation of proteolysis (Costa *et al*.,

2007). Also, it might reveal that the elimination rate of the drug, in the insect, exceeded the absorption rate (Kharbouche *et al*., 2008). Herein, clonazepam could be detected significantly from the empty puparium of *L. cuprina* in all the treated groups (from 0.7 to 6 mg/mL), compared to the negative and positive controls (Fig. 3). Previous results revealed the high sensitivity of the protein carbonyls analysis in detecting clonazepam in *S. argyrostoma* (Afifi *et al*., 2022 a).

The current results showed an increase in the scavenging percentage along with all the concentrations of clonazepam and all the tested immatures (Fig. 4). Furthermore, using the DPPH inhibition percentage revealed the successful detection of the drug in all stages and in the empty puparium in comparison to the negative control (i.e. the naïve carcass which died without any injections). This revealed the possibility of using the empty puparium of *L. cuprina* in case of late discovery of a corpse. Also, the antioxidants of the larval instars (1st, 2nd, and 3rd instars) elevated significantly in relation to the positive control, in all drug concentrations. A lot of studies used DPPH in antioxidant analyses, for example, assessing various pharmaceuticals activities (Parmar *et al*., 2010), investigating tea polyphenols in detoxification of free radicals (Wei *et al*., 2016), and assessing the antioxidant ability of the Malathion-exposed black soldier fly (Close *et al*., 1997). To the best of our knowledge, this was the first study concerning the feasibility of using DPPH antioxidant analysis in the forensic entomotoxicology field (Wei *et al*., 2016; Afifi *et al*., 2022 b)

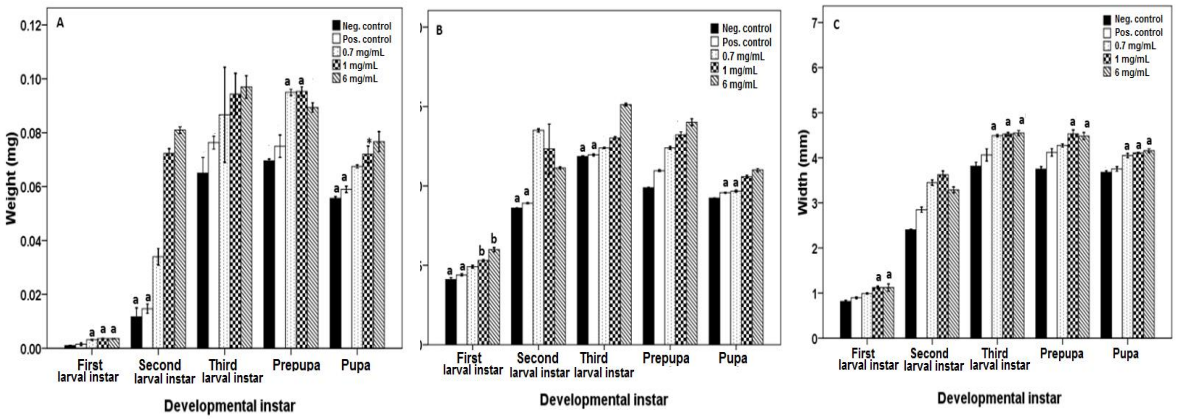

Fig. 1. Impact of different concentrations of Clonazepam (0.7, 1, or 6 mg/mL), with respect to negative and positive controls, on the morphological measurements of larval, prepupal, and pupal stages of *Lucilia cuprina*: (A) weight (mg), (B) length (mm) and (C) width (mm). Data expressed as median and standard deviation (SD). Median values marked with the small letters were insignificantly different (p>0.05) regarding clonazepam concentration.

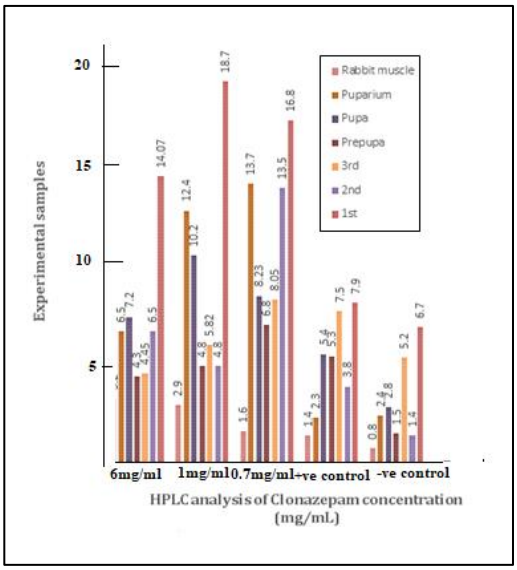

Fig. 2. Detection of Clonazepam concentration in larval, prepupal, pupal, and empty puparium tissues of *Lucilia cuprina*, which fed on different concentrations of Clonazepam (0.7, 1, or 6 mg/mL), with respect to negative and positive controls. Data expressed as median and standard deviation (SD). Median values marked with the small letters were insignificantly different (p>0.05) regarding clonazepam concentration.

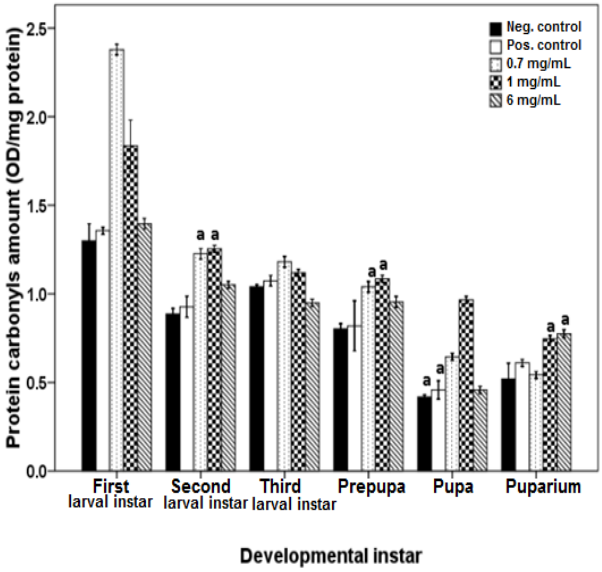

Fig. 3. Detection of protein carbonyls amount (OD/mg protein) in larval, prepupal, pupal, and empty puparium tissues of *Lucilia cuprina*, which fed on different concentrations of Clonazepam (0.7, 1, or 6 mg/mL), with respect to negative and positive controls. Data expressed as median and standard deviation (SD). Median values marked with the small letters were insignificantly different (p>0.05) regarding clonazepam concentration.

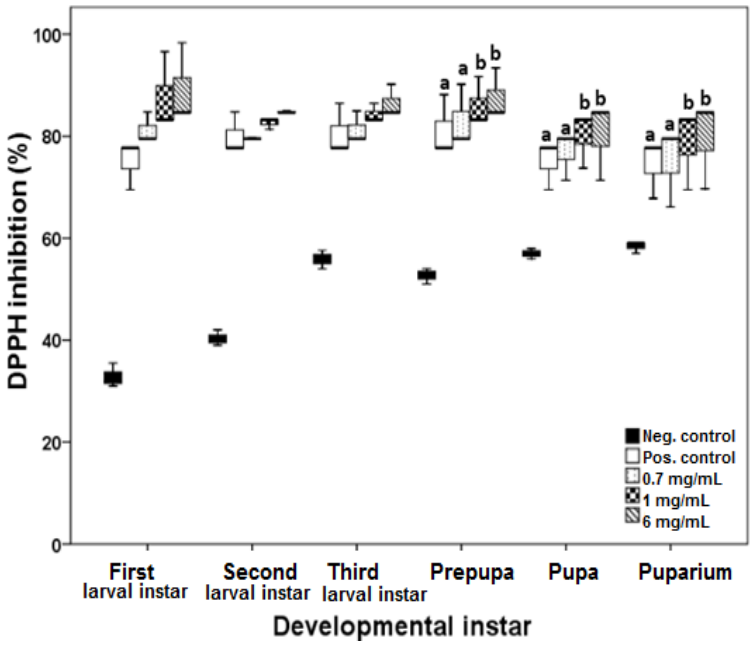

Fig. 4. Detection of α,α-diphenyl-β-picrylhydrazyl (DPPH) inhibition (%) in larval, prepupal, pupal, and empty puparium tissues of *Lucilia cuprina*, which fed on different concentrations of Clonazepam (0.7, 1, or 6 mg/mL), with respect to negative and positive controls. Data expressed as median and standard deviation (SD). Median values marked with the small letters were insignificantly different (p>0.05) regarding clonazepam concentration.

Conclusion

The concentration of clonazepam in injected rabbits directly affected *L. cuprina* growth rate and significantly increased their weight, length, and width. Moreover, HPLC-MS and protein carbonyl amount method were effective in detecting the drug in all insect's developmental stages. DPPH was very sensitive and effective in recording the biochemical changes in the drug-injected rabbits, through the insect's immature feeding habits on carcasses. Additionally, the empty puparial case of *L. cuprina* demonstrated its reliability in detecting clonazepam at the investigated concentrations.

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تقنيات جديدة وتقليدية للكشف عن الكلونازيبام في *Lucilia cuprina* (Diptera: Calliphoridae)

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المستخلص

تستخدم أدوية البنزوديازيبين، ولا سيما كلونازيبام، كعلاج مساعد لإدارة الصرع والسيطرة على النوبات. ومع ذلك، فإن سوء استخدام هذه الأدوية يمكن أن يكون قاتلاً. وفي مثل هذه الحالات، يمكن لعلم الحشرات المساعدة من خلال إجراء اختبارات مرضية وسمية محددة للعثور على أدلة جنائية لبقايا الدواء داخل الجثة حتى عندما تكون الجثة متحللة للغاية. تقيّم هذه الدراسة فعالية طريقتين جديدتين، وهما قياس كربونيل البروتين واستخدام دي بي بي اتش (DPPH)، في الكشف عن كلونازيبام في لوسليا كوبرينا حيث تم تغذية يرقات الحشرة على أنسجة عضلات الأرانب المحقونة بكلونازيبام. كما تم مقارنة هذه الطرق بالتقنية التحليلية التقليدية "كروماتوغرافيا السائل عالية الأداء-طيف الكتلة" .(HPLC-MS) . بالإضافة إلى ذلك، تم تقييم تأثير الدواء على نمو الحشرة من خلال قياس البيانات المورفومترية في المراحل اليرقية والشرنقة واليرقة. أظهرت النتائج زيادة كبيرة في الخصائص المورفولوجية (الوزن والطول والعرض) في جميع المجموعات المعالجة بالدواء، مقارنة بالمجموعات الضابطة السالبة و / أو الموجبة. علاوة على ذلك، تسبب الدواء في ارتفاع ملحوظ في مستويات كربونيل البروتين، مما يشير إلى تلف أكسدة في المجموعات المعالجة مقارنة بالمجموعات الضابطة. كما زاد كلونازيبام بشكل كبير من نسبة تثبيط DPPH مقارنة بالضوابط السالبة. كشفت تقنية HPLC-MS بنجاح عن ارتفاع كبير في تركيز كلونازيبام في جميع عينات الحشرة المعالجة. علاوة على ذلك، أثبتت الشرانق الفارغة للحشرة صحتها في الكشف عن الدواء.

الكلمات المفتاحية : علم الحشرات الجنائي، علم السموم ، البنزوديازيبينات، الإجهاد التأكسدي، البيانات النمائية.