MEASURING PHOSPHINE IN BOTH POSTMORTEM TISSUES OF ALUMINUM PHOSPHIDE INTOXICATED CORPSES & ASSOCIATED CHRYSOMYA ALBICEPS LARVAE

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ABSTRACT

Objectives: Aluminum phosphide (ALP) has been extensively used as an economical and effective insecticide, rodenticide, and fumigant. The active ingredient of ALP is phosphine (PH3), the ease availability of which can lead to mass suicidal poisoning with high mortality. Exposure to PH3 will give rise to entire damage in the human body. The magnitude of ALP poisoning and its numerous related deaths in man with lack of entomotoxicology screening methods and quantitative methods for this toxin prompted us to undertake this study. **Aim of work:** The current study aimed to measure phosphine (PH3) concentrations by a quantifying method in postmortem (PM) specimens (blood, lung, liver, small intestine and 1st & 6th day 3rd instar larvae of chrysomya albiceps) from intoxicated corpses to find a correlation between the PH3 concentration in PM specimens & larvae to help in the detection of ALP toxicity as a cause of death. Also, this study aims to explore the effects of ALP on C. albiceps life cycle and bio morphological changes to estimate the accurate postmortem interval (PMI). **Methodology:** This study reviewed 10 chemically confirmed cases were included and the effect of ALP on C. albiceps life cycle through studying the parameters & the time duration of its different stages. Also, phosphine analysis in both PM specimens (blood, lung, liver and small intestine) and (1^{st} & 6^{th} day) 3^{rd} instar C. albiceps larvae using GC- MS. **Results:** there was significant increase in means values of larval lengths and weights were observed for most measurements at different stages which reared on ALP poisoned tissues comparing to those of the control cases. Also, there was marked acceleration in the average development duration of C. albiceps life cycle stages reared on the studied ALP poisoned cases tissues as comparing to those of the control cases. On the other hand, the method described here for the analysis of PH3 is rapid, sensitive, and free of all chromatographic interferences. This method provides acceptable selectivity and stability for PH3 determination. The analyte was found to be linear within the calibration range (0.2 up to 8 µg/mL). **Conclusion:** ALP caused acceleration of the life cycle duration of C. albiceps and changes in the biometric diameters of its larvae. These results can aid in estimating the time and the cause of death. According to GC- MS analysis, the method described here presents a sensitive and selective approach for the quantification of PH3 at accurate and reliable values within a concentration range of 0.2–8 µg/mL for forensic applications.

Keywords: Aluminum phosphide (ALP), GC- MS, C. albiceps, entomology.

INTRODUCTION

Forensic entomology is a branch of biology that involves the use of insects in legal investigations ***(Njau et al., 2015).*** Immature stages, mostly fly larvae, are collected from and around a corpse at crime scenes.

Insects play an important role in tissue decomposition, so many species have become

useful in forensics ***(Byrd and Tomberlin, 2019),*** which studies and uses data about insects and their development to help solve criminal cases. Various entomological pieces of evidence, such as eggs, larvae at various stages, pupae, imagoes, or indirect traces of insect existence (e.g., exuviae, feces), appear on a corpse over time after death. A careful examination of the collected material, patterns of entomological succession, and the rate at which insects develop can aid in determining the post-mortem interval (PMI), which is the time between death and the body's disclosure ***(Pujol-Luz et al., 2008; Bhardwaj et al., 2020 & Jales et al., 2020).***

Postmortem interval can be calculated by determining insect species and estimating their stage of development. Insects give an accurate PMI estimate due to their predictable developmental rate and their invasion sequence on carrion ***(Njau et al., 2015).*** They can also help in predicting the cause of death, translocation of dead bodies, and may help in identifying unknown corpses ***(Roy et al., 2021).***

At crime scenes, all traces left by criminals and victims may be important for clarification of facts, regardless of type, quality, or quantity of material recovered**.** This material may include evidence from the inside insects' gut. Carrion-feeding maggots (fly larvae) store food in their crop, an organ located at the anterior end of the gut ***(Stoffolano, 2019).***

Entomotoxicology, which is one of the newest aspects of forensic entomology, involves toxicological and molecular examinations of insects to help in elucidating the cause of death. The analysis of larvae found in corpses can assist in the detection of drugs and toxins present in the corpses ***(Hodecek et al., 2020).*** Concentrations of drugs and toxins in larvae could be correlated to concentrations in tissues consumed by the larvae, giving a valuable clue about the cause of death ***(Campobasso et al., 2004).***

The first insects to colonize a corpse are true flies of the Calliphoridae family and their larvae have the largest share in active decomposition ***(Márquez-Grant, 2012).*** In optimal thermal conditions, thousands of larvae are capable of decomposing soft tissues very quickly, before the discovery of the corpse and collection of samples for toxicological analysis ***(Campobasso et. al.,2004).*** If tissues are not available, it is possible to identify chemical compounds in the insects feeding on the corpse (larvae, pupae, adult forms) or even in the exuviae or excreta of those insects ***(Gagliano-Candela and Aventaggiato, 2001).*** Larvae feeding on a tissue containing toxic substances introduce these substances into their metabolic system. The detected substances are of various types (organic and inorganic), e.g., medicines, poisons, drugs or pesticides.

The main method of identification of xenobiotics (medicines, drugs, alcohol) in larval tissues is chromatography that allows separation and analysis of the chemical composition of the mixture of various compounds. Among the methods used in entomotoxicology are gas chromatography (GC), thin layer chromatography (TLC), high pressure liquid chromatography-mass spectrometry (HPLCMS), as well as radioimmunoassay (RlA) and gas chromatography-mass spectrometry (GC-MS) ***(Campobasso et. al., 2004).***

Also, it is critical to keep in mind that drugs or toxins (as well as insecticides) found in decomposing corpses can affect the developmental cycle of insects. Direct ingestion of toxins by insects via tissue or transmission through the food chains of necrophagous/predatory insects can eventually impair the accuracy of PMI estimation ***(Campobasso et al., 2004; George et al., 2009; Abd Al Galil et al., 2021).*** This is why it is necessary to have a thorough understanding of the relationship between the rate of development of a particular insect species and the concentration of a toxic substance in the insects' body and corpse tissues ***(Vasconcelos et al., 2016).***

Chrysomya albiceps (C. albiceps) (Diptera: Calliphoridae) one of the most forensically important flies found in the Afro-tropical and oriental regions ***(Tantawi et al., 2018).*** This species is recognized as among the first wave of insects that visit and colonize human cadavers. Currently, this species is of great importance for medical and veterinary forensics which utilized to indicate the PMI in human deaths. Adults are attracted to corpses within minutes after death. The flies detect carcasses primarily by odor, and attraction

varies with the degree of decomposition. Adult females oviposit on corpses/carcasses, and their larvae feed on the fresh decomposing tissues ***(François et al., 2017)***.

Aluminum phosphide (ALP), known as grain tablets in Egypt, is a solid fumigant pesticide & easily available under brand name "celphos " tablet. It is considered to be an ideal pesticide because of its cheapness, efficiency and easy availability in the market and is widely used as a grain preservative worldwide ***(Amro A. Saleh and Mohammed G. Makhlof, 2018).*** None of the patients who had ingested more than three tablets survived. The average time interval between ingestion of AlP and death is 3 h (1–48 h), 95% of the patients die within 24 h (***Kalawat et al., 2014)***.

Aluminum phosphide is capable of releasing phosphine gas (PH3) if it comes in contact with moisture in the air. Phosphine can easily be distributed into all tissues and excreted in the urine and expelled through the lungs (***Hashemi-Domeneh, et al., 2016).***

Since ALP is a major cause of suicidal poisoning in many countries especially in Egypt with high mortality rate (***El-Sarnagawy, 2017),*** some deaths may be discovered lately in an advanced stage of decomposition, no organ or tissue samples are viable to screen for toxins. So, we can use of entomotoxicology to detect toxins that may not discovered during the analysis of decomposing body tissues and fluids of a body after death (***Joshi and Kumar, 2020)***.

So, the magnitude of ALP poisoning and its numerous related deaths in man with lack of entomotoxicology screening methods and quantitative methods for this toxin prompted us to undertake this study.

MATERIALS AND METHODS

- **Ethical Considerations***:*

- The Ethics Committee of Scientific Research, Faculty of Medicine, Benha University for this study was obtained (MD 5- 7- 2020)***.***

- **Number of cases in the present study,**

- 10 medicolegal autopsy cases of aluminium phosphide were studied. All the study cases were confirmed to be of Aluminum phosphide poisoning on chemical analysis of the viscera in the Forensic Science laboratory.
- 10 control cases were selected in which the cause of death was either thermal or mechanical injuries.
- All the cases were collected from Forensic Medicine Authority, Benha, Qalyubiyah, Egypt.

- **Required data:**

- Efforts were made to obtain detailed and accurate history regarding the time and quantity of ingestion of Aluminum phosphide and the time of death.
- Chemical analysis of the viscera was done and those cases where viscera are positive for alcohol or any other toxin were not included in the present study.

A. Entomotoxicology experiment:

1**- Flies' collection and identification**

- All the flies used in this study were collected throughout Faculty of Sciences, Benha, Qalyubiyah Governorate, Egypt in December 2021 ***(Figure 1)***. To ensure that all the individuals collected belonged to Chrysomya albiceps (C. albiceps), they were reared in the lab of Benha Faculty of Science for nine generations before completion of the study .
-Identification was done at each generation, 5–10 males and females were selected for identification morphologically based on taxonomic keys of Carvalho and Mello-Patiu .(2008)
- Following the method described in Pimsler et al. (2014), species determination was then confirmed by a 100% by estimating genome sizes for all samples, to detect whether they were associated with a sequence or not.

2- **Rearing Flies *(Figure 2):***

- Post-mortem (PM) samples (viscera and liver) were obtained from the corpses used for rearing C. albiceps larvae on them.
- For each ALP poisoned or control case, thirty adults (20 females & 10 males) flies were reared in a separate cage inside at room temperature, maintained at 30°C, 70% humidity, and with 14: 10 (Light: Dark) h photoperiod. The cages were protected with an external net curtain to avoid the entry of other insect species ***(Whitworth, 2006).***

- Adult females oviposited eggs on PM specimens. The eggs observed from each cage were then transferred gently into a larva-rearing box containing PM specimens. Hatching of eggs was checked every three hours.
- Larvae, pupae and adults were also checked every twelve hours until the emergence of adults (***Baeeri et al., 2013).***
- Larvae were allowed to feed and collected at each stage.
- The larval development was monitored until pupation. When pupae were observed, they were transferred using forceps into a new container containing sterile fine sand; each container was then placed into an individual insect cage. Once the adult flies emerged, a mixture of 10% (w/v) sugar solution was provided as adult food.

3- <u>Sampling of larvae for biomorphic studies:</u>

- Collections and sampling were performed randomly among the carcasses. The larval sample collection of both groups was occurred daily at the same time. The collected larvae were immersed in hot water for 30 seconds and then transferred in 70% ethanol for preservation (Adams and Hall, 2003).

4- <u>Measurements of body weights and lengths of each stage of larvae</u> were carried out regularly *(Figure 3& 4).*

- The weight was recorded by using a sensitive electrical balance with a sensitivity of nearest 1 milligram (mg). The length was measured to the nearest millimeter (mm) using simple rulers with mm marks clearly visible.

5- **The total time taken by each stage of C. albiceps life cycle was recorded.** The temperature and humidity were recorded daily with the help of Hygro-thermometer clock.

Fig (1): Flies' collection by rearing the flies on rabbit corpse.

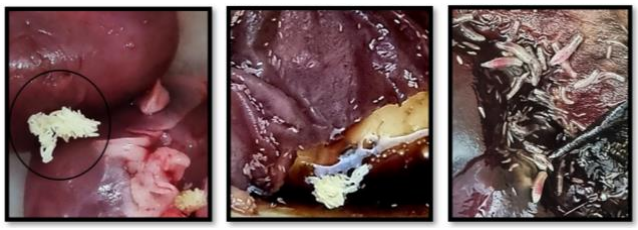

Fig (2): Observation of the different stages of C. albiceps on ALP PM samples.

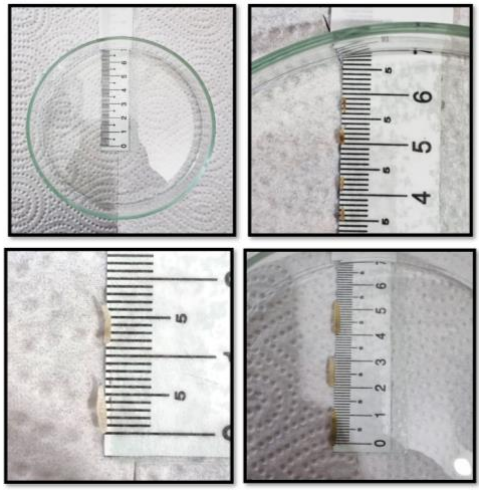

Fig (3): Measuring body lengths of each stage of larvae using simple rulers with mm marks clearly visible.

Fig (4): Measuring body weights of each stage of larvae using a sensitive electrical balance with a sensitivity of nearest 1 milligram (mg).

B. Gas chromatography couples with mass spectrometry (GC- MS) analysis:

- **Reagents:**

Concentrated sulfuric acid (Fisher, reagent grade) was diluted using distilled water. Analyzed grade toluene was used to dissolve the PH3 .

- **Instrumentation:**

A rotary mixer (Millipore Corp., Bedford MA) at 30 rpm was used to mix the sample matrix with the H2SO4 and to extract the PH3 into the toluene layer. Calibrated solvent pipettor pumps were used to dispense toluene (10 mL minimum

capacity) and sulfuric acid (90 mL minimum capacity). The bottles were centrifuged in a Beckman model TJ-6centrifuge. A capillary gas chromatograph (Hewlett-Packard Co., model 5890) equipped with a nitrogen-phosphorus detector (NPD) was used in GC analysis.

- **Sample collection for GC-MS analysis:**

  - ***Post mortem samples*** (Heart blood, lung, liver and small intestine) were received from both control & ALP poisoned cases, frozen in sealed vials at -20 °C and stored under these conditions till the time of GC-MS analysis.

  - ***Sampling of larvae:*** The collected 3rd instar larvae (1st day & 6th day) were received from both control & ALP poisoned cases, frozen in sealed vials at -20 °C and stored under these conditions till the time of GC-MS analysis to detect PH3 concentration.

- **Sample preparation:**

For the sample preparation, 40 micrograms (μg) of zinc powder were placed uniformly in the bottom of a 10-mL headspace vial, and 1 mL of biological fluid (whole blood) or 1 g of tissues, then immediately, 10 mL of toluene solvent was added to trap any phosphine gas, followed immediately by 90 mL of 20% sulfuric acid were added successively. Then, each vial was immediately sealed with a crimp cap and silicone/polytetrafluoroethylene (Teflon) septum. The samples in the bottles were then shaken for 1-1.5 h on shaker. After mixing, all bottles were centrifuged for 2 min at 1500-1800 rpm. For injection the toluene phase was transferred from sealed bottles to the sealed GC vials using a syringe. PH3 was then extracted from the HS by solid-phase microextraction (SPME) with GC/MS analysis by selected ion monitoring (SIM) of m/z 31, 33 and 34.

- **Method validation:**

To generate PH3 for the calibration standards, the reduction reaction and replacement reaction of sodium hypophosphite according to Eq. (1) was performed (***Lu et al., 1987).***

$2NaH_2PO_2 + 4Zn + 5H_2SO_4 \rightarrow 4ZnSO_4 + Na_2SO_4 + 4H_2O + 2PH_3\uparrow$ (1)

For this, 31.8 mg of $NaH_2PO_2 \bullet H_2O$ was weighed and dissolved in 10 mL of water to obtain an aqueous stock solution (The initial concentration of the primary standard was taken into consideration in preparing the necessary dilutions). The mixtures were mixed for a minimum of 30 min on the rotary mixer. The working standards were prepared by stepwise 1:10 dilutions in water and stored in a desiccator.

The validation procedure was performed according to ASB Standard Practices for Method Validation in Forensic Toxicology ***(Standard, 2017).*** Selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity and stability parameters were studied for validation.

- Blank matrices (whole blood, lung, liver and small intestine) from 10 different sources were studied to determine selectivity.
- Potential interferences were investigated at the expected retention time of PH3.
- A series of decreasing concentrations of spiked blood was evaluated to determine the LOD and LOQ.
- The linearity parameter was investigated by analysis of seven calibration points for concentrations of the analytes of 0.1, 0.2, 0.5, 1, 2, 5, and 8 μg/mL.
- Stability determinations comprised freeze-and-thaw cycle stability (three cycles) and short-term stability (4 and −20 °C) in plasma. An acceptance interval was applied for the mean stability sample concentration as compares to control samples mean (freshly prepared).

The stability experiments were performed for low and high concentrations (1 and 6 μg/mL) with six repetitions.

- The samples to be analyzed were entered into a sequence on the chromatography data station. The retention time (RT) of PH3 on the GC/MS is approximately (1 min). LOD for this method is 0.1 μg/ mL, and LOQ for this method is 0.2 μg/ mL.

RESULTS

At the end of the experimental period all cases were subjected to the following studies:

I. Entomotoxicology experiment:

- **Observations:**

There were some color changes in the larval stages of c. albiceps fed on ALP poisoned cases' tissues, which were observed more darkened in color comparing to those reared on the control cases. Also, the skeletons of 3rd instar larvae reared on ALP poisoned cases were more fragile than those of the control cases by touch.

- **Biomorphic studies:**

Tables (1, 2) and figures (5, 6) show mean values of larval lengths and weights at different stages (1st instar, 2nd instar, 3rd instar (1st, 2nd, 3rd, 4th, 5th & 6th day)), for each fed on control & ALP poisoned cases collected at the same time. Significant increase in means values of larval lengths and weights were observed for most measurements at different stages.

The means of lengths of the larvae reared on ALP poisoned cases and those of the control cases are presented in table (1) & figure (5,7). Larvae of the ALP poisoned cases showed an increase from 1.8 ± 0.57 mm at 1st instar stage to 11.8 ± 0.908 mm at 3rd instar (6th day) with the control recording length increase from 2.2 ± 0.274 mm to 11.5 ± 0.612 mm at the same larval stages.

The mean lengths value of 1st instar larvae reared on control cases (2.2) was more than those of ALP poisoned cases (1.8). But, the mean lengths value of 2nd instar larvae reared on ALP poisoned cases (3.5) was more than those of control cases (2.9).

Also, the mean lengths value of 3rd instar larvae (1st day) reared on control cases (6.1) was more than those of ALP poisoned cases (6). But, the mean lengths value of 3rd instar larvae (2nd day) reared on ALP poisoned cases (12.8) was more than those of control cases (11.2).

As well, the mean lengths value of 3rd instar larvae (3rd day) reared on ALP poisoned cases (14.1) was more than those of control cases (11.4). The mean lengths value of 3rd instar larvae (4th day) reared on ALP poisoned cases (13.6) was more than those of control cases (11.5). Also, the mean lengths value of 3rd instar larvae (5th day) reared on ALP poisoned cases (13) was more than those of control cases (11.5). And, the mean lengths value of 3rd instar larvae (6th day) reared on ALP poisoned cases (11.8) was more than those of control cases (11.6).

There is a significant increase in mean values of length of (2nd instar, 3rd instar (2nd day), 3rd instar (3rd day), 3rd instar (4th day) & 3rd instar (5th day)) larvae in ALP poisoned cases as compared to those of the control cases. There is no statistically significant difference between ALP poisoned cases and the control cases at (1st instar, 3rd instar (1st & 6th day).

The weight means of the larvae reared on ALP poisoned and the control cases are presented in table (2) & figure (6). The results show that the mean weight of larvae of C. albiceps reared on ALP poisoned cases was 0.64 ± 0.207 mg at 1st instar stage and reached 32.76 ± 5.36 mg at 3rd instar (6th day).

The mean weights value of 1st instar larvae reared on control cases (0.8) was more than those of ALP poisoned cases (0.64). But, the mean weights value of 2nd instar larvae reared on ALP poisoned cases (1.26) was more than those of control cases (1.1).

Also, the mean weights value of 3rd instar larvae (1st day) reared on control cases (5.68) was more than those of ALP poisoned cases (3.22). But, the mean weights value of 3rd instar larvae (2nd day) reared on ALP poisoned cases (30.76) was more than those of control cases (22.46).

As well, the mean weights value of 3rd instar larvae (3rd day) reared on ALP poisoned cases (41.74) was more than those of control cases (26.38). The mean weights value of 3rd instar larvae (4th day) reared on ALP poisoned cases (46.6) was more than those of control cases (30.74). Also, the mean weights value of 3rd instar larvae (5th day) reared on ALP poisoned cases (37.58) was more than those of control cases (31.12). And, the mean weights value of 3rd instar larvae (6th day) reared on ALP poisoned cases (32.76) was more than those of control cases (32.18).

This is significantly more than most of weight means of control larvae stages that had weight means of 0.8 ± 0.122 mg at 1st instar stage and 32.18 ± 6.79 mg at 3rd instar (6th day).

- **Estimation of developmental duration of C. albiceps life cycle:**

Table (3) showed the average development duration of C. albiceps life cycle stages reared on the studied cases tissues which is markedly accelerated in those of ALP poisoned cases as comparing to those of the control cases.

The average duration of eggs (from oviposition till hatching) was (11 h.) for those of ALP poisoned cases and (24h.) for those of the control cases under the same environmental conditions (table 3).

The average duration of 1st larval instar was (12 h.) for those of ALP poisoned cases and (23h.) for those of the control cases under the same environmental conditions. For 2nd larval instar, development time in ALP poisoned cases was nearly similar (13 h.) to 1st instar, also (25.5 h.) for those of the control cases (table 3).

The average development time for feeding phase of 3rd larval instar was the longest which was 145.5 h. & 215.5 h. for ALP poisoned & control cases respectively (table 3).

In pupal stage, the average duration of development markedly accelerated in those reared-on ALP poisoned cases comparing to those of the control cases (142.5h. & 168 h.) respectively at the same conditions (table 3).

According to the PMI of the control cases, Table (3) showed that the eggs persisted 24 hours. After hatching eggs, the 1stt instar larva took 23 hours to become 2nd instar larva stage. The PMI duration since egg laid was 47 hours. The 2nd instar larva took 25 hours 30 minutes to reach 3rd instar larva and the PMI duration was 72 hours 30 minutes. The 3rd instar larva took 215 hours and 30 minutes, PMI duration was 288 hours. The pupal stage took 168 hours to become adult fly emerged. So, the total duration of whole life cycle of C. albiceps reared on the control cases was 456 hours.

Also, for the PMI of the ALP poisoned cases, Table (3) showed that the eggs persisted only for 11 hours. After hatching eggs, the 1stt instar larva took 12 hours to become 2nd instar larva stage. The PMI duration since egg laid was 23 hours. The 2nd instar larva took 13 hours to reach 3rd instar larva and the PMI duration was 36 hours. The 3rd instar larvae took 145 hours and 30 minutes, PMI duration was 181 hours and 30 minutes. The pupal stage took 142.5 hours to become adult fly emerged. So, the total duration of whole life cycle of C. albiceps reared on the ALP poisoned cases was 324 hours.

So, C. albiceps larvae cultured on tissues of ALP poisoned cases developed much faster 24 hours than those reared on control tissues after egg hatching till the emerging of the adult flies after approximately 324 hours. This reduces the duration of the whole development process by 132 hours comparing to the duration of the C. albiceps cycle on the control tissues.

Table (1): Means of larval lengths of C. albiceps that reared on (liver & viscera) of ALP poisoned cases in comparison to the control cases at different stages:

stages	Mean ± SD of Length in (mm)				P Value (T test)
	Control cases		ALP poisoned cases		0.01**
	Mean ± SD	Range	Mean ± SD	Range	
1st instar	2.2 ± 0.274	2- 2.5	1.8 ± 0.57	1- 2.5	0.24*
2nd instar	2.9 ± 0.224	2.5- 3	3.5 ± 0.5	3- 4	0.035**
3rd instar (1st day)	6.1 ± 0.224	6- 6.5	6 ± 0.354	5.5- 6.5	0.31*
3rd instar (2nd day)	11.2 ± 0.447	11- 12	12.8 ± 0.975	11.5- 14	0.004**
3rd instar (3rd day)	11.4 ± 0.894	10 -12	14.1 ± 0.57	13.5- 15	0.002**
3rd instar (4th day)	11.5 ± 0.866	10- 12	13.6 ± 0.894	12.5- 14.5	0.018**
3rd instar (5th day)	11.5 ± 0.612	11- 12.5	13 ± 0.707	12- 13.5	0.004**
3rd instar (6th day)	11.6 ± 0.822	10.5- 12.5	11.8 ± 0.908	10.5- 13	0.39*

P-value < 0.05 considered significant
(*) Non-Significant.
(**) Significant.

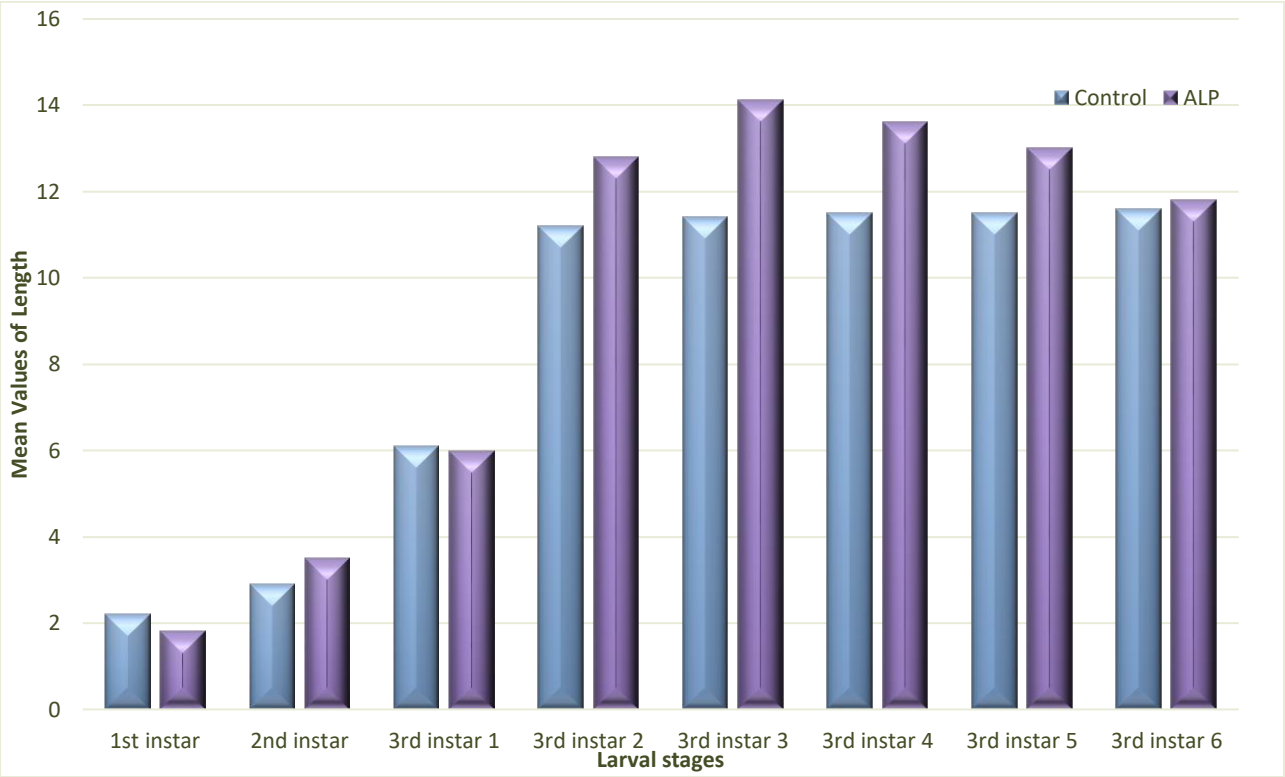

Fig (5): Bar chart showing mean values of larval lengths of C. albiceps that reared on (liver & viscera) of ALP poisoned cases in comparison to the control cases at different stages.

Table (2): Means of larval weights of C. albiceps that reared on (liver & viscera) of ALP poisoned cases in comparison to the control cases at different stages:

stages	Mean ± SD of Weight in (mg)				P Value (T test)
	Control cases		ALP poisoned cases		0.03**
	Mean ± SD	Range	Mean ± SD	Range	
1st instar	0.8 ± 0.122	0.6 – 0.9	0.64 ± 0.207	0.4 – 0.9	0.088**
2nd instar	1.1 ± 0.394	0.8 – 1.7	1.26 ± 0.336	0.9 – 1.8	0.3*
3rd instar (1st day)	5.68 ± 0.672	4.8 – 6.4	3.22 ± 0.481	2.6 – 3.6	0.003**
3rd instar (2nd day)	22.46 ± 3.51	18.8 – 23.7	30.76 ± 3.012	27.3 – 33.4	0.003**
3rd instar (3rd day)	26.38 ± 6.46	17.5 – 34	41.74 ± 5.03	33.8 – 47.4	0.014**
3rd instar (4th day)	30.74 ± 8.03	23.8 – 43.5	46.6 ± 11.55	35.1 – 62.1	0.056*
3rd instar (5th day)	31.12 ± 4.14	28.5 – 37.8	37.58 ± 4.21	33.9 – 44.4	0.036**
3rd instar (6th day)	32.18 ± 6.79	22.9 – 39.6	32.76 ± 5.36	27.9 – 41.6	0.44*

P-value < 0.05 considered significant
(*) Non-Significant.
(**) Significant.

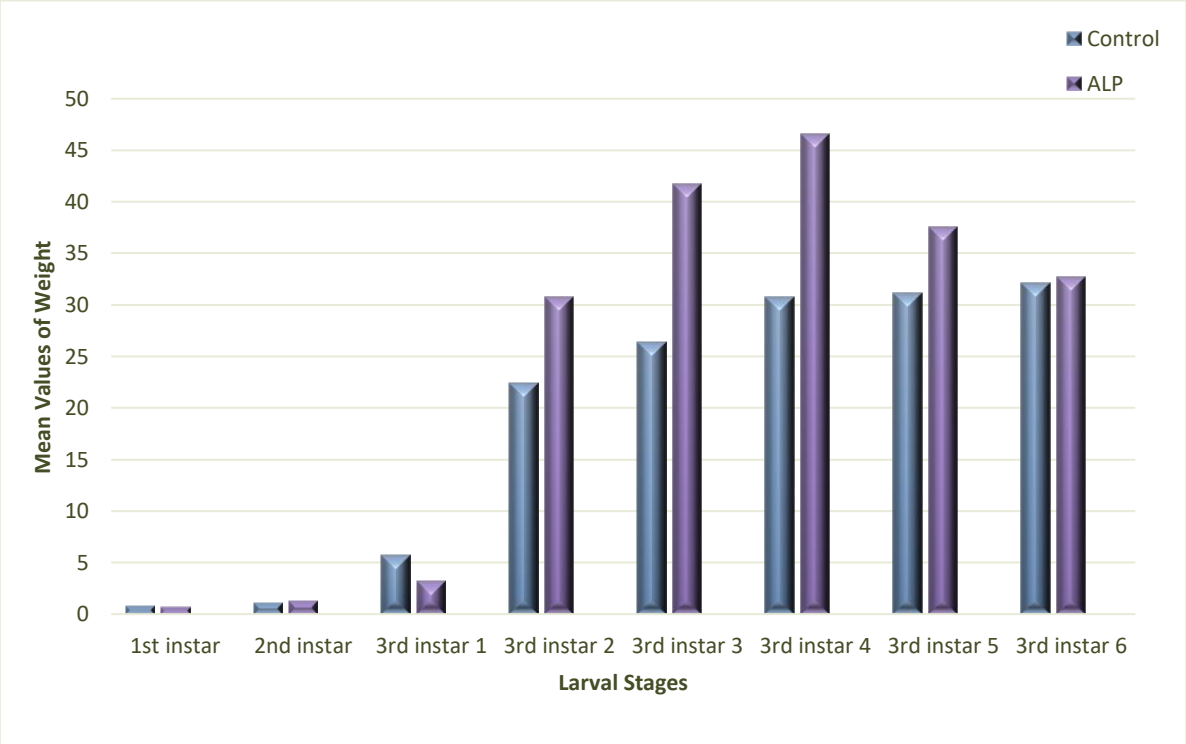

Fig (6): Bar chart showing mean values of larval weights of C. albiceps that reared on (liver & viscera) of ALP poisoned cases in comparison to the control cases at different stages.

Table (3): The average duration of different C. albiceps developmental stages that reared on (liver & viscera) of ALP poisoned cases in comparison to the control cases at the same environmental conditions:

Life stages		Average Duration (H)			
		Control cases		ALP poisoned Cases	
		Duration (H)	PMI (H)	Duration (H)	PMI (H)
Eggs		24	-	11	-
Larva	1st instar	23	47	12	23
	2nd instar	25.5	72.5	13	36
	3rd instar	215.5	288	145.5	181.5
Pupa		168	456	142.5	324
Total duration		456 h (19 days)		324 h (13.5 days)	

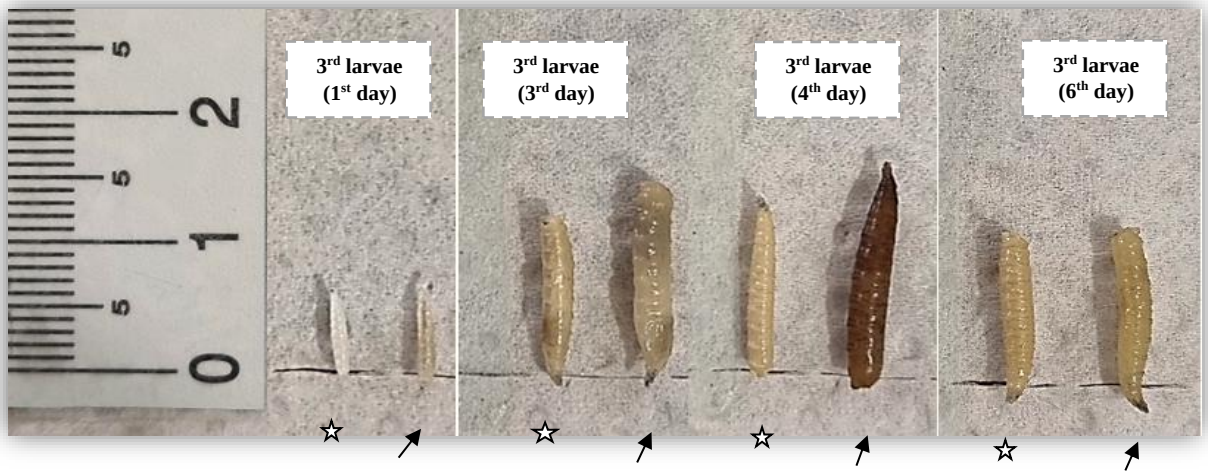

Fig (7): Comparison of body lengths of *C. albiceps* 3rd larvae (1st, 3rd, 4th & 6th days) of control (*) & ALP cases (arrow) at the same environmental conditions.

II. GC- MS analysis:

The method described here for the analysis of PH3 is rapid, sensitive, and free of all chromatographic interferences. It is possible to analyze a batch of 20 samples and 3-6 recovery check spikes in 1 day (8 h).

➢ Method validation:

- **Selectivity:**

Blank heart blood, lung, liver and small intestine samples from 5 individuals were investigated for co-eluting chromatographic peaks. No interference peak was observed at the retention time of PH3, demonstrating that this method provides acceptable selectivity for PH3 determination. The total ion current chromatograms of all monitored ions for spiked and blank blood sample were shown in figure (8). The air peak was removed in the solvent delay.

- **Linearity, LOD, and LOQ:**

The analyte was found to be linear within the calibration range (0.2 up to 8 µg/mL), using the mean values of the duplicate analyses, calibration curves were checked for variance homogeneity (F-test) and for linearity (Mandel test). The linear regression is described by the following equation: y = 278.29x- 18.399 (R-square = 0.999). The calibration curve was measured twice, before and after the biological samples. Limit of detection (LOD) and quantification (LOQ) were 0.1 µg/ mL and 0.2 µg/ mL for PH3 in human blood sample.

- **Stability:**

The stability data was shown in Table (4). Blood sodium hypophosphite was stable after

24 h at 4 °C or after 7 days at −20 °C. The freeze thaw stability was within acceptance range.

- **Deceased samples analysis:**

The biological samples (Heart blood, lung, liver and small intestine) were sampled and analyzed by GC–MS for PH3. The analytical results for total PH3 concentration in cases are shown in **Table (5)**.

No interference peak was observed at the RT of PH3 (1 min) in this method. While the PM samples taken from non-exposed bodies (control subjects) were tested negative for PH3.

Regarding PH3 concentrations in blood and tissue samples from the ALP poisoned cases, the highest concentrations of PH3 was in the liver specimens followed by small intestine, blood & lung in the same order, then the 3rd instar larvae.

The mean values of PH3 concentrations in the blood, lung and small intestine were 1.02 µg/ml, 0.71 µg/g and 1.18 µg/g respectively. These mean values were not significantly different from each other as shown in **table (6).**

But there is significantly increase in the mean value of PH3 concentrations which detected in the liver samples (1.76 µg/g) comparing to those of other biological samples (blood, lung and small intestine) as shown in **table (6).**

The mean values of the concentration of PH3 in the 3rd instar larvae of C. albiceps (1st day, 6th day) were 0.54 µg/g and 0.37 µg/g respectively. These mean values were significantly lower than ALP poisoned cases tissues and blood as shown in table **(6,7).** On the other hand, there is a positive correlation between the PH3 concentration in 3rd larvae (1st day & 6th day) and ALP poisoned cadaver blood and tissues as shown in **figure (9).**

Also, there is significantly decrease in the mean value of PH3 concentrations in 3rd instar of C. albiceps (6th day) comparing to those of 3rd instar of C. albiceps (1st day) as shown in **table (6,7).**

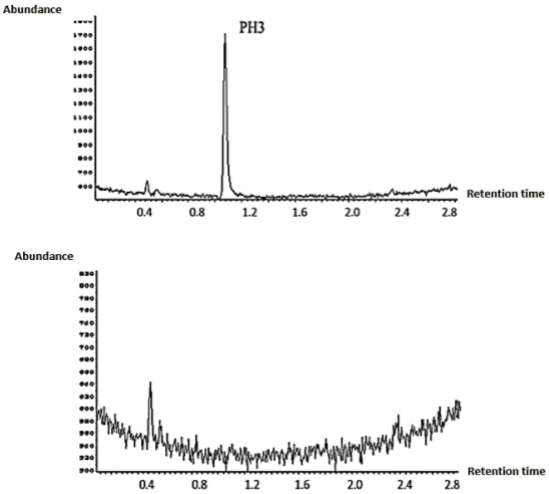

Fig (8): Total ion current chromatograms of spiked (PH3 1 µg/mL) and blank human blood sample.

Table (4): Recovery% showing the stability of sodium hypophosphite in blood:

Levels (µg/ mL)	Recovery percentage (%)		
	4 °C for 24 h	−20 °C for 7 days	Three frozen/thaw cycles
1	85.2	88.2	81.9
6	94.3	92.4	96.7

Table (5): The analytical results of the total PH3 concentration in biological fluids and tissues (µg/mL or µg/g):

Subjects	Blood	Lung	Liver	Small Intestine
1	**ND**	**0.56**	**0.2**	**0.27**
2	**0.2**	**0.3**	**0.9**	**0.54**
3	**ND**	**0.35**	**0.2**	**0.4**
4	**ND**	**ND**	**0.35**	**0.4**
5	**1.4**	**0.3**	**1.9**	**0.73**
6	**0.7**	**0.64**	**2.1**	**0.93**
7	**2**	**ND**	**0.9**	**1.3**
8	**3.1**	**0.3**	**4.7**	**3.2**
9	**ND**	**0.84**	**2.1**	**1.8**
10	**2.8**	**0.8**	**4.2**	**2.2**

ND non-detected.

Table (6): The mean values of analytical results of the total PH3 concentration in different days of 3rd larval stages of C. albiceps (µg/g) that reared on ALP poisoned cases:

ALP Poisoned cases	Mean values of PH3 in 3rd instar (1st day) (µg/g)	Mean values of PH3 in 3rd instar (6th day) (µg/g)
1	0.45	0.33
2	0.22	0.17
3	0.38	0.26
4	0.11^{+}	0
5	0.52	0.39
6	0.53	0.38
7	0.3	0.24
8	1.1	0.8
9	0.57	0.34
10	1.2	0.75

Positive sign (+) denotes < LOQ.

Table (7): Mean values of PH3 concentrations in different biological samples of ALP poisoned cases in comparing to those of the control cases (µg/mL or µg/g):

Sample	Control cases (µg/mL or µg/g)	ALP poisoned cases (µg/mL or µg/g)	Significant pairs &P	
Blood (A)	**0**	**1.02**	**C≠A**	**0.033 (*)**
			C≠B	**0.003 (*)**
Lung (B)	**0**	**0.71**	**C≠D**	**0.026 (*)**
			C≠E	**0.007 (*)**
Liver (C)	**0**	**1.76**	**F≠A**	**0.038 (*)**
			F≠B	**0.03 (*)**
Small Intestine (D)	**0**	**1.18**	**F≠C**	**0.005 (*)**
			F≠D	**0.008 (*)**
3rd instar (1st day) (E)	**0**	**0.54**	**F≠E**	**0.002 (*)**
			D≠E	**0.008 (*)**
3rd instar (6th day) (F)	**0**	**0.37**	**D≠B**	**0.01 (*)**

P-value < 0.05 considered significant
(*) Significant.

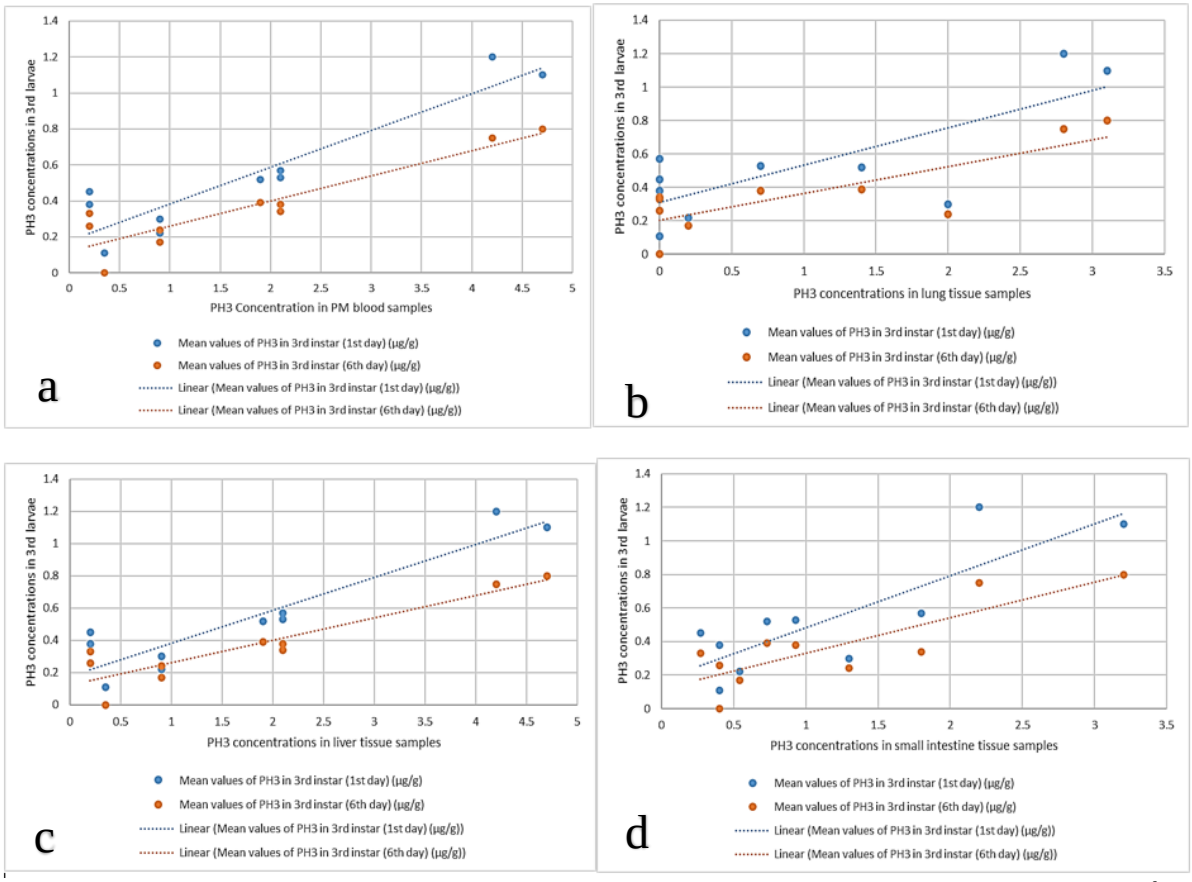

Fig (9): (a) A scatter chart showing the correlation between PH3 concentrations in blood and 3rd larvae of c. albiceps samples. (b) A scatter chart showing the correlation between PH3 concentrations in lung tissue and 3rd larvae of c. albiceps samples. (c) A scatter chart showing the correlation between PH3 concentrations in liver tissue and 3rd larvae of c. albiceps samples. (d) A scatter chart showing the correlation between PH3 concentrations in small intestine tissue and 3rd larvae of c. albiceps samples.

DISCUSSION

In forensic entomology, necrophagous insects are useful for determination of the PMI, post mortem transfer, and presence of toxins.

The deaths related to drug use, or to accidental or deliberate use of poisons or toxic substances, explain the interest in the field of entomotoxicology. This branch of forensic science, which is especially useful when a corpse is in an advanced state of decomposition or skeletonized and its tissues and fluids are no longer suitable for toxicological analyses, deals with the investigation of the effects of drugs and toxins present in human tissues on the biology of necrophagous insects. Seminal studies have indicated that the presence of toxic substances can affect the development of necrophagous insects, for instance altering the development time in larvae of blow flies, muscid flies and flesh flies (***Gião et al., 2017)***.

Insects feeding on corpse tissues absorb the same substances as in a deceased body before death **(*Amendt et al., 2009)*.** In this study, we selected chrysomya albiceps (c. albiceps) which is a common initial colonizer of carcasses in Afrotropical regions, Oriental regions, Central and South America, and Southern Europe. C. albiceps is well-known as among the first wave of faunal succession on human cadavers, which makes it a valuable tool for the estimation of minimum PMI ***(Salimi et al., 2018).***

The results of the present study showed that c. albiceps larvae is sensitive to the action of ALP, with morphological changes that can disrupt the configuration of species in a system characterized by necrophagous insects.

During the current study color changes were noticed. These changes were darkening in color of the larvae which appeared from the 1st instar, gradually increased and persist till the end of the larval stage (6th day of the 3rd instar) among c. albiceps reared on ALP poisoned cases. This darkening may be referred to the tissues that c. albiceps were reared on, which was very congested.

Also, results of this study showed more fragility in the skeletons of the 3rd instars larvae reared on ALP poisoned cases comparing to those of the control cases. This may be explained by ***Gião et al. (2017)*** who reported that A chemical in the larval environment may affect the individuals that feed on it, and also their offspring.

The current study showed a significant increase in the mean values of larval lengths and weights in the different stages of the larvae that collected from ALP poisoned tissues as compared to those of the control cases at the same time.

This may be explained as insect growth is affected by temperature, humidity, competition and most importantly, food. The presence of drugs and other toxic substances in food substrates can directly alter the growth and development rate of these insects. Depending on the type of drug, growth and development can be accelerated or retarded ***(Salimi et al., 2018)***. When substrate is available, may stimulate faster feeding by individual maggots, and the metabolic rate may be markedly increased, which could result in increased body weight and length ***(Bosly, 2021)***.

In contrast, ***El-Ashram et al. (2022)*** reported that the larvae of both experimental groups showed a regular body length gain over during the experiment, although their development was slowed down in the ALP group.

In general, the present study showed changes in the average development duration of all c. albiceps life cycle stages reared on the ALP poisoned cases tissues which is markedly accelerated as comparing to those of the control cases under the same environmental conditions.

The average duration of eggs (from oviposition till hatching) was (11 h.) for those of ALP poisoned cases and (24h.) for those of the control cases. Also, the average duration of 1st larval instar was (12 h.) for those of ALP poisoned cases and (23 h.) for those of the control cases. For 2nd larval instar, development time in ALP poisoned cases was nearly similar (13 h.) to 1st instar, and (25.5 h.) for those of the control cases. The average development time for 3rd larval instar was the longest which was 145.5 h. & 215.5 h. for ALP poisoned & control cases respectively. In pupal stage, the average duration of development markedly accelerated in those reared-on ALP poisoned cases

comparing to those of the control cases (142.5h. & 168 h.) respectively.

So, C. albiceps larvae cultured on tissues of ALP poisoned cases developed much faster 24 hours than those reared on control tissues after egg hatching till the emerging of the adult flies after approximately 324 hours. This reduces the duration of the whole development process by 132 hours comparing to the duration of the C. albiceps cycle on the control tissues.

Toxins affect the growth rate of larvae developing on the corpses. Many studies have assessed the impact of drugs/pesticides on the development of fly species in terms of forensic entomology ***(Mahat et al., 2012 & Magni et al., 2018).*** The vast majority of drugs accelerate the development of larvae ***(Carvalho and Mello- Patiu, 2008 & Ferrari et al., 2008).***

However, other insecticides as DEET - N, N-Diethyl-meta-toluamide and malathione can delay the development of fly larvae ***(Shelomi et al., 2012).*** Also, ***El-Ashram et al. (2022)*** was opposed the current results and reported that C. albiceps larvae development was slowed down in the ALP group.

That contrast may be explained by the same species could react differently toward two molecules belonging to the same family ***(Elshehaby et al., 2019).*** In the other hand, the effects of exposure to toxic substances are too complex to evaluate in the field, because they usually induce various types of interactions between the toxicant and density dependence, mitigating or enhancing the effect on the population ***(Halide, 2018).***

This information should be taken in consideration when estimating the PMI. Results can be helpful to establish evidence while determining the cause of death. Analysis of the life cycle duration can aid in estimating the time of death. So, further studies on the effect of these drugs on the life cycle of c. albiceps need to be conducted in order to help to estimate the PMI accurately.

On the other hand, the present study tested the selectivity & stability parameters for blank blood, lung, liver, small intestine and larva samples which all provided acceptable selectivity for PH3 determination in addition to acceptance range for the freeze thaw stability.

Indeed, a linear relationship was discovered between the injected sample concentration and the area of the generated peak. This method therefore allows concentration measurements in blood & tissue samples at accurate and reliable values within a concentration range of 0.2–8 μg/mL.

According to the current results, the method described here presents a sensitive and selective approach for quantifying PH3 for forensic applications especially the protocol was done on real cases of ALP intoxication.

Detection of phosphide exposure in an early human forensic case involved combining either blood or homogenized tissue with 10% sulfuric acid in a HS vial, 30 s vortex mixing, then injection of 1 mL of the sample into a gas chromatograph equipped with a thermionic nitrogen–phosphorus detector ***(Chan et al., 1983).***

Tiwary et al. (2005) and Du et al. (2019) described a method in which 1g of crop or gizzard contents was similarly treated with sulfuric acid in HS vials, and phosphine was then extracted from the HS by solid-phase microextraction (SPME) with GC/MS analysis by selected ion monitoring (SIM) of m/z 31, 33 and 34 with a detection limit of 1 μg/g. This detection limit was considered sufficient for veterinary diagnostic purposes.

More rigorous and near-complete evolution of PH3 gas was described by ***Yan et al. (2017).*** Those authors combined 1 g or 1 mL of animal sample with 40 μg of zinc powder and then treated this with 20% sulfuric acid; as a result, they could claim sensitivities down to 0.2 μg/mL using GC/MS.

Perz et al. (2015) also very sensitively applied HS-GC/MS SIM of ions m/z 31, 33 and 34 to dried foodstuffs and achieved limits of detection of 0.1 μg/kg; high sensitivity was achieved by the use of an absorbent-filled liner and cryo-cooling.

The current study observed that the toxicological analysis confirmed that PH3 can affect all the studied body organs (blood, lung, liver and small intestine) with more distribution in the liver. As there was significantly increase

in the mean value of PH3 concentrations which detected in the liver samples (1.76 µg/g) comparing to those of other biological samples (blood, lung and small intestine). On the other hand, the mean values of PH3 concentrations in the blood, lung and small intestine were not significantly different from each other. From the current analytical results, we can conclude that liver was a good choice for the determination of PH3. On the other hand, blood may not be a perfect choice in the analysis of PH3 poisoning due to the negative results obtained in 40% of the studied cases.

These findings were in a harmony with ***Yan et al. (2017)*** who reported that the toxicological analysis of their animal experiment confirmed that PH3 can affect all organs in the body, more distributed in the brain, heart, and the liver which was a good choice for the determination of PH3.

In contrast, ***El-Ashram et al. (2022)*** reported that the highest concentrations of PH3 were found in the kidney, blood, and liver of rabbits in the ALP-treated group, at 50.79 µg/g, 44.44 µg/mL, and 34.60 µg/g, respectively. These mean values were not significantly different from each other.

In the current study, the highest PH3 concentrations that detected in the liver PM specimens may be explained by ***Saleki et al. (2007)*** who reported that PH3 is rapidly absorbed throughout the gastrointestinal tract after ingestion and it is partly carried to the liver by the portal vein. It is known that PH3 can cause liver dysfunction, especially after the first day of poisoning.

On the other hand, PM blood specimen in the current study was not a good choice for PH3 analysis. This is may be explained by ***Elshehaby et al. (2017)*** who said that although, antemortem blood concentrations of drugs can be used to estimate the amount of administered drug, this is not always possible in PM cases. The PM blood concentrations do not accurately reflect the blood concentrations at the time of death mainly due to PM redistribution. After death, drugs are redistributed to the surrounding tissues either by diffusion through blood vessels or by trans-parietal diffusion towards the surrounding organs.

To our best knowledge, our present work is one of the few studies that details analytical results of PH3 after fatal ALP intoxication.

Few literature reports have examined the toxicological results of PH3 after fatal PH3 intoxication. A 25-year-old man was found dead after ingestion of ALP. PH3 concentrations were found in the stomach contents (0.2 µg/g), nose smear (0.56 µg/g), and small intestine (0.28 µg/g) by an HS-GC/ NPD method with a LOD of 0.65 ng/g, while the analysis of urine, femoral and heart blood, liver, kidney, bile, and brain revealed negative results for PH3 ***(Musshoff et al., 2008).***

Chan et al. (1983) measured the PH3 concentration in PM blood (0.5 ng/mL), liver (3 ng/g), and stomach contents (3 µg/g) of a man who had ingested tablets containing ALP using HS-GC/NPD.

Also, ***Anger et al. (2000)*** reported that a 39-year-old man committed suicide by ingesting ALP, and PH3 was determined in the brain (94 mL/g), liver (24 mL/g), kidney (41 mL/g), adrenals (2.4 mL/g), and heart (0.9 mL/g) by HS-GC-MS.

The results of the current work demonstrated that the mean values of the concentration of PH3 in the 3rd instar larvae of c. albiceps (1st day, 6th day) were 0.54 µg/g and 0.37 µg/g respectively. These mean values were significantly lower than ALP poisoned cases tissues and blood. On the other hand, there is a positive correlation between the PH3 concentration in 3rd larvae (1st day & 6th day) and ALP poisoned cadaver blood and tissues.

These results are in line with ***El-Ashram et al. (2022)*** who reported that the concentration of AlP in the third larval instar of C. albiceps was 11.24 µg/g, and the value was significantly lower than in the rabbits' tissues.

These results may be explained by some scientists who found a direct correlation between the toxin/drug concentration in larvae and cadaver tissues. Still, others found no correlation or postulated that xenobiotic concentrations found in larvae could be significantly lower than in body tissues ***(FOX, 2011).***

For example, ***Introna et al. (1990)*** found that morphine concentrations in C. vicina larvae feeding on decomposing liver tissues of deceased humans who died of morphine poisoning were highly correlated with post-mortem tissue concentrations.

Campobasso et al. (2004) investigated the correlation between xenobiotic concentrations in the human liver and larvae of the Calliphoridae family Lucilia sericata (L. sericata) feeding on the tissue. Opiates, cocaine, barbiturates, and antidepressants were among the toxins tested. All of the xenobiotics found in L. sericata bodies were in lower concentrations than in human tissues. Thus, the concentration of many substances in insect tissues is often lower than in the source. This is because many toxins are partly metabolized and gradually excreted by insects (***El-Ashram et al., 2022)***. Also, this explained the significant decrease in the mean value of PH3 concentrations in 3rd instar of C. albiceps (6th day) comparing to those of 3rd instar of C. albiceps (1st day) in the current study.

CONCLUSION

In conclusion, this study demonstrated that ALP caused acceleration of the life cycle duration of C. albiceps and changes in the biometric diameters of its larvae. These results can aid in estimating the time and the cause of death. According to GC- MS analysis, the method described here presents a sensitive and selective approach for the quantification of PH3 at accurate and reliable values within a concentration range of 0.2–8 μg/mL for forensic applications.

RECOMMENDATIONS

- Raising public awareness about its toxicity and fatality through the media is an urgent requirement.
- A thorough study is required to comprehend the predisposing aspects and mechanism of action in order to assess and establish an effective treatment in such cases.
- Treatment options should be widely available in hospitals.
- It should be illegal to store it or sell it, and only people with permission should be able to use, store, or sell it.
- GC- MS analysis protocol of the present study to be acceptable method to measure PH3 in PM samples, the Precision and accuracy parameters are needed to be tested following the Standard Practices for Method Validation in Forensic Toxicology.
- Application of GC- MS analysis protocol to real cases of aluminum phosphide intoxication provided reliable results.
- Additional studies using different species of Calliphoridae and Sarcophagidae are needed as there may be different responses to the drug as the evidence held by insects in a crime scene is not prone to destruction.

CONFLICTS OF INTEREST

There are no conflicts of interest declared by the authors.