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RESEARCH ARTICLE

Effect of coumarin on carrion decomposition and its secondary bioaccumulation in *Chrysomya rufifacies* larvae

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ABSTRACT

The presence of chemical substances can interfere with the insect activities, affecting the decomposition duration of carrion, thus under-estimating the minimum post-mortem interval. This study investigates the effects of coumarin an active ingredient in anti-coagulant rodenticide, on carrion decomposition and secondary bioaccumulation in fly larvae tissue. Twenty-four rat carcasses were divided into control (CR) and coumarin-treated (TR) groups, and their decomposition stages were observed under identical environmental conditions. Results indicate that coumarin delayed the decomposition by approximately four days, primarily due to its impact on insect arrival and larval activity. *Chrysomya rufifacies* (Macquart, 1843) (Diptera: Calliphoridae) was the predominant species in both groups, with slower larval feeding on TR carcasses prolonging the transition from active to advanced decay. Secondary bioaccumulation of coumarin in third-instar larvae was detected using Gas Chromatography-Mass Spectrometry (GC-MS). These findings highlight coumarin's potential to alter insect succession patterns and its implications for minimum post-mortem interval (minPMI) estimation in forensic investigations.

Keywords: coumarin; forensic entomology; decomposition; *Chrysomya rufifacies*; bioaccumulation.

INTRODUCTION

Entomotoxicology application is useful in forensic investigation as it provides clues in intoxication-related death, especially when there are no residual cadaveric tissues and body fluids (Chakroborty & Sharma, 2023). The entomotoxicology field focuses on two problems, which are, when and how the victims die. The former problem can be investigated by studying the growth rates of necrophagous arthropods for minimum post-mortem interval (minPMI) estimation, while the latter is an alternative (Harvey *et al*., 2016). Sometimes, the only way to acquire chemically related information is from insects instead of body fluids and rotten tissues, which can interfere with toxicological analysis (Sari *et al*., 2023).

The presence of chemical substances on carrion or a dead body is often not well-documented and can be challenging to detect. The chemical product may result in delayed colonisation and the absence of the first arrival of necrophagous species on the carcass/cadaver (Campobasso *et al*., 2001). Moreover, previous studies have shown that chemicals ingested by carrion-feeding arthropods through their food can influence insect development (Abajue & Ewuim, 2018; Al-Keridis *et al*., 2022). These scenarios can cause underestimation of minPMI as the calculation is based on the growth rate of insects (Zou *et al.*, 2013).

The World Health Organization (WHO) has identified chemical substances as a major public health issue, contributing significantly to morbidity and mortality. Worldwide, intentional pesticide ingestion results in over 168,000 deaths annually, accounting for 20% of all suicides, with most cases reported from developing countries (Gunnell *et al*., 2007; Mew *et al*., 2017) In Malaysia, according to National Poison Centre from 2006 to 2015, about 10% of reported cases was due to rodenticides poisoning (Kamaruzaman *et al*., 2020).

Anticoagulant rodenticides (AR) are the most widely used rodenticides and also referred to as Anti-Vitamin K's (AVKs) to control rodents such as rats and mice. It interferes with the biosynthesis of vitamin K-dependent coagulant factors in the liver which are important in coagulation. The active compound of AR is coumarin and its derivatives i.e., warfarin, brodifacoum and flocoumafen (van Sittert & Tuinman, 1994; Hommerding, 2022). Rodent control is vital for public health and hygiene, as rodents spread diseases, contaminate food, and damage buildings. While rodenticides are a common choice alongside biological control, they pose risks of primary and secondary poisoning to humans, animals, and the environment (Erofeeva *et al*., 2023).

Although several studies on the effects of insecticides and herbicides on insect colonization and carrion decomposition have been carried out previously (Yan-Wei *et al*., 2010; Abd El-Bar & Sawaby, 2011; Jales *et al*., 2021), the study on the effects of rodenticides' active compounds or its derivatives are limited. This study investigates, the effects of coumarin on insect colonization and the detection of the accumulated coumarin within *Chrysomya rufifacies* larvae feeding on coumarin intoxicated-rat carcasses.

MATERIALS AND METHODS

Study site

The study was conducted at Kampung Sri Kandong, Jalan Taman Negara Kubah, Kuching, Sarawak, Malaysia (1”38’03”N, 110”09”35”E), 24 m above sea level (Figure 1). The study site was a secondary forest, consisting of small trees and dense ground vegetation. Based on the environmental data provided by the Meteorological Department of Malaysia (MET Malaysia), the annual ambient temperature in Kuching ranged from 23 to 33°C (annual mean: 27°C). Kuching receives a lot of rain even in the driest month, where the average rainfall accumulates at 2435.1 mm yearly. In this study, the ambient temperatures and relative humidity were recorded *in situ* using a data logger (Extech RHT20, Malaysia).

Pesticide preparation

Coumarin (Sigma, USA) was prepared according to oral LD_{50} dosage for rats by diluting it in warm (80°C) distilled water to allow the granule to dilute completely. The oral LD_{50} dosage for rats was 0.293 g/kg (NPIC, 2019).

Animal model preparation

Twenty-four male albino rats (*Rattus norvegicus*), aged 3–4 months and weighing approximately 180–200 g, were used as animal models in this study. The rats, sourced from a local rodent farm in Kuching, Sarawak, were healthy upon delivery. They were housed individually in cages for at least 72 hours before the experiment followed methods by Smith (2009).

The rats were divided into two groups which were labelled as control (CR) and coumarin-treated group (TR). Each group consists of four rats. Before the pesticide administration, the rats were fasted overnight to increase the bioavailability and absorption of the pesticides inside the gastrointestinal system (Smith, 2009). Each group consisted of four individual rats and were given distilled water (CR) and coumarin (TR) through an oral administration (force feed) procedure following the animal ethics guideline. The substance was allowed to absorb in the rats’ bodies for at least one hour before being euthanized by cervical dislocation. The substance administration and euthanization procedure was performed by a trained medical laboratory technologist and this study has obtained animal ethics approval from Animal Ethic Committee Universiti Malaysia Sarawak (UNIMAS/AEC/F07/051).

Fieldwork experiment

After preparation, the carcasses were wrapped in double plastic bags and immediately transported to the study site in a cooler box. At the study site, the rat carcasses within the same group were placed one meter apart from each other, and with 20 meters between each group. Visits to the carcasses were conducted twice daily, approximately at 9 am and 4 pm. Entomological samples were collected during each visit for the colonization study. In the field, the carcasses were covered with slotted plastic baskets with approximately 1 cm openings to prevent scavenger predation while still allowing insect access. Each basket was secured to the ground by placing a brick on top to keep it in place.

The experiment was repeated in three separate trials with each trial lasting about one month and an interval of at least two weeks between trials. Each trial of the experiment was conducted at the same location.

Insect collection and species identification

Adult insects at the study site were monitored and collected for preservation and species identification. Adult insects attracted to the carcasses were captured with sweep nets; while crawling insects were collected with forceps or by hand. Captured adults were killed in a killing jar containing cotton which was soaked with ethyl acetate, which preserves the insects’ morphology. The insects were pinned and stored in insect boxes for identification. Species identification was based on guides by Clearwater (1981), Nazni *et al*. (2011), Creedy & Mann (2011), and Nemes & Price (2015).

Fly larvae colonizing the carcasses were collected using forceps. Approximately 20 larvae were collected daily during the active decay stage, which lasted for three days. From the total population of collected larvae, twenty individuals were preserved in 70% ethanol and later mounted for species identification. Another twenty larvae were reared in the laboratory until they emerged as adults for species identification. The remaining larvae were used for subsequent toxicological analysis.

The mounting procedure followed methods by Heo *et al*. (2008). A ventral mid-line incision was made on the larval body, which was then soaked in 10% potassium hydroxide (KOH) (Merck, USA) for 24 hours until the internal tissues were completely cleared. Any remaining opaque gut or fatty tissues were removed using fine forceps and dissecting needle. Cleared larvae were rinsed after removal from KOH and soaked in 10% acetic acid (HmbG, Malaysia)

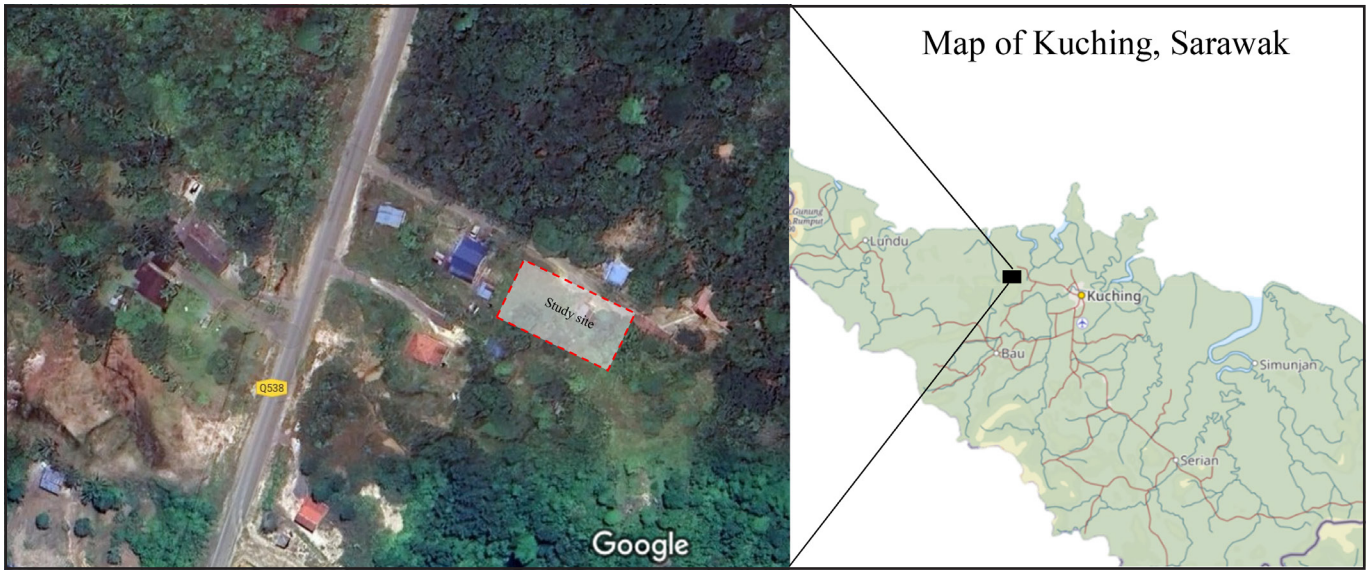

Figure 1. The map shows the study site at Kampung Sri Kandong, Jalan Taman Negara Kubah, Kuching, Sarawak, Malaysia (1°38’03”N,10°09’35”E). (Source: Adapted from Google Map 2024).

for 30 minutes. The larvae were then rinsed again with distilled water and gradually dehydrated by soaking in a series of ethanol solutions (HmbG, Malaysia) at concentrations of 30%, 50%, 70%, and 90% for 30 minutes each.

At the final dehydration step, the larvae were soaked in 100% ethanol for one hour with two changes of ethanol. After the final ethanol was removed, the larvae were soaked for 30 minutes in clove oil (Fisher Chemical, UK). Each larva was subsequently placed on a clean glass slide and washed with xylene (Sigma, USA). After the removal of excess xylene, two drops of Canada balsam (Sigma Aldrich, USA) were applied to cover the entire larva, a cover slip was placed on the larva to preserve the larva specimen. Once the prepared slides were dried, the larvae were observed under a compound microscope (Optika, Italy). The larval characteristics were observed and species identification was based on Szpila (2009) and Akbarzadeh *et al*. (2015).

Detection of coumarin bioaccumulation

Third instar larvae collected from the carcasses were used to detect secondary bioaccumulation of coumarin using Gas Chromatography-Mass Spectrometry (GCMS). One gram (18-20 individual larvae) of predominant third instar larvae was cleaned and killed in hot water (80°C) for 5 seconds. The individual larvae were kept frozen in a -20°C freezer for subsequent toxicological analysis. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction method was employed, following the procedures described by Vetter *et al*. (2017) with some modifications. Frozen larvae samples were thawed and homogenised manually using mortar and pestle. Ten millilitres of homogenised samples, ceramic homogenisers (Agilent Technologies, USA) and 10 ml acetonitrile were placed in a 50 ml centrifuge tube. The ceramic homogenisers are added to facilitate breaking up matrix and salt agglomerates for higher extraction recoveries of target analytes. The mixture was vortexed for one minute and subsequently 6.5 g of QuEChERS Extract Pouch (Agilent Technologies, USA) was added and vortexed for another one minute before being subjected to centrifugation for five minutes at 2,500 *g* in room temperature.

Before analysis, the supernatant undergoes clean-up process using SPE kit (Agilent Technologies, USA). One ml of the supernatant was transferred into a 1.5 ml microcentrifuge tube, vortexed for one minute and centrifuged at 9,500 g at room temperature. The cleaned supernatant was finally transferred into a GCMS vial and analysed. The positive control was prepared by diluting analytical grade coumarin in acetonitrile in a concentration of 0.5 mg/ml.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC-MS method for coumarin detection followed the procedure by Vetter *et al*. (2017). The samples of each trial were analysed using Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu QP GC-2010 Plus) in the following condition. One microlitre of the sample was injected into GC-MS at a temperature of 270°C and flow rate of 1.4 ml/min of helium as carrier gas. The separation was carried out using a non-polar column, cross-linked BPX-5 (30 m x 0.25 mm x 0.25 µm film thickness composed of non-polar 5% phenyl methyl siloxane) with the following oven program: The initial temperature was 50°C for 2 minutes, then increased up to 150°C (25°C/min), ramping up to 240°C (10°C/min) and finally up to 300°C (50°C/min) where the final temperature was held for one minute. Data was collected in the selected ion monitoring (SIM) technique at selected ions (m/z 50-250). The identification of the analyte was performed by matching the peak obtained from the database in the National Institute of Standard and Technology (NIST) library based on the values of the retention time, molecular formula, molecular weight and concentration.

Data analysis

The comparison of environmental data between trials was analysed using One-way ANOVA with a significance level of 0.05 (SPSS ver. 27). The entomological data was tabulated and the relative abundance was analysed using the following formula:

Relative abundance of species (%)

$$Relative\ abundance = \frac{The\ abundance\ of\ one\ species\ (ni)}{Total\ all\ species\ counted\ (N)} \times 100$$

RESULTS

Environmental parameters at the study site

Throughout the three intervals of the study period, the average daily surface temperatures ranged from 26.4±1.2 (Mean±S.D) to 28.2±1.2°C. The highest accumulated rain recorded during the first trial of the experiment from January to February of 2021 was 542.6 m which is reflected by a significantly lower average temperature recorded during that period. The least accumulated rain was recorded during the third trial from September to October 2021 with a total of 5.4 mm, accompanied by significantly lower average relative humidity, 82.8 ± 5.5% during the same time. The summary of environmental data is presented in Table 1.

One-way ANOVA analysis was conducted to compare the environmental parameters between trials. The analysis showed there is a significant difference in daily surface temperatures [F (2,48) = 11.21, P<0.001] and relative humidity [F (2,48) = 10.29, P<0.001]. Multiple comparison analysis, Tukey post-hoc test showed the environmental parameters during the third trial (September-October) was significantly higher (28.2±1.2°C) than the average temperatures and humidity during the first (26.4±1.2°C) and second (27.3±1.0°C) trials of the experiment which was conducted in January and March 2021, respectively.

Composition of forensically important insect

There was a total of 997 individual insects associated with decomposing rat carcasses belongs to four orders and eight families were collected over the study period (Table 2). The number of insects visiting the TR group (n=541) of carcasses was slightly higher, than the CR group (n=456). However, the difference in insect abundance between these two groups was not significant [t (4) = -0.574, P= 0.597]. *Chrysomya rufifacies* was the predominant fly colonising on both groups of carcasses (74.2%), followed by *Chrysomya*

Table 1. Environmental data recorded over study period showing the average temperatures and humidity from three trials of experiments

Intervals	Mean daily surface temperatures (°C) (Mean±S.E.)			Average relative humidity (%)	Accumulated rain (mm)
	Maximum	Minimum	Average		
21st Jan – 15th Feb	29.1 ± 0.5	23.7 ± 0.2	26.4 ± 1.2	89.7 ± 4.1	542.6
3rd – 20th March	31.2 ± 0.4	23.2 ± 0.2	27.3 ± 1.0	88.1 ± 4.0	283.5
22nd Sept – 11th Oct	32.5 ± 0.6	24.0± 0.2	*28.2 ± 1.2	*82.8 ± 5.5	65.4

*Statistical significance P<0.05.

Table 2. The composition and abundance of insects collected on decomposing rat carcasses for control (CR) and coumarin-treated (TR) group in three trials of experiment

Order	Family	Genus/Species	Total number of individual species in each group			Relative abundance (%)
			CR	TR	Total	
Diptera	Calliphoridae	*Chrysomya rufifacies*	365	376	742	74.2
		Chrysomya megacephala	16	71	87	8.7
		Hemipyrellia ligurriens	48	9	53	5.7
		Lucilia sp.	3	0	2	0.3
		Hypopygiopsis violacea	1	0	1	0.1
	Sarcophagidae	*Sarcophaga* sp.	1	5	6	0.6
	Muscidae	*Ophyra chalcogaster*	9	23	32	3.2
		Atherigona orientalis	7	11	18	1.8
Coleoptera	Scarabaeidae	*Onthophagous* sp.	4	36	40	4.0
	Hydrophilidae	Unidentified	1	3	4	0.4
	Staphylinidae	*Aleochara* sp.	1	7	8	0.8
Total			**456**	**541**	**997**	**100**

megacephala (Fabricius, 1794) (8.7%) and *Hemipyrellia ligurriens* (Wiedemann, 1830) (5.7%). Other blowflies such as *Lucilia* sp. and *Hypopygiopsis violacea* (Macquart, 1835) were the least number of blow flies collected which were only present in CR carcasses.

Besides blow flies, *Sarcophaga* sp. (Diptera: Sarcophagidae) and muscid flies (Diptera: Muscidae) were also recovered on the carcasses. Among coleopterans, *Onthophagous* sp. (Coeloptera: Scarabaeidae) was the most abundant species recovered from rat carcasses. A higher number of *Onthophagous* sp. was recorded in TR rat carcasses (n=36) compared to CR carcasses (n=4). Other beetles collected was belonging to family Staphylinidae and Hydrophilidae. A few other species, such as Dermaptera and Hemiptera, were also observed targeting the larvae colonizing the carcasses during the decay stage, but only on TR carcasses.

Carrion decomposition and colonization pattern

The decomposition process of rat carcasses consists of five stages: fresh, bloated, active decay, advanced decay, and dry remains. The decomposition of TR carcasses took approximately 12 days, while the CR group took approximately eight days to complete. The fresh stage began immediately after the rats were euthanized. Within 15 minutes of exposure at the study site, the first insect observed on CR carcasses was *Chrysomya megacephala*. The first insect arrival on TR carcasses occurred after 24 hours, with *Chrysomya rufifacies* being the first visitor.

The bloated stage started when the carcasses' abdomens became distended, which occurred after 24 hours. During this stage, there was an intensifying odour of decay. On CR carcasses, *C. megacephala*, *C. rufifacies*, *Hypopygiopsis violacea*, *Hemipyrellia ligurriens*, *Lucilia* sp., and *Sarcophaga* sp. were observed. Oviposition predominantly occurred at natural orifices, although some species also deposited eggs on the distended abdomens of carcasses, likely attracted by the release of fluids and gases. By day 2, TR carcasses had also entered the bloated stage. *Chrysomya rufifacies* and beetles, including *Onthophagus* sp., *Aleochara* sp., and members of the family Hydrophilidae were present. Beetles were observed crawling on the carcasses and congregating beneath them when lifted. Fly eggs were also detected on TR carcasses during this stage.

The active decay stage began 24 hours after the bloated stage. During this phase, the carcasses' abdomens began to collapse, and the internal organs and tissues decomposed into body fluids, which were purged out through natural orifices and openings created by fly larvae. In CR carcasses, larvae of *C. rufifacies* and *C. megacephala* were observed actively colonising the natural orifices area. On TR carcasses, more adult flies (*Hy. violacea*, *H. ligurriens*, *Lucilia* sp., and *Sarcophaga* sp.) arrived, while larvae of *C. rufifacies* dominated, followed by *C. megacephala*. Initially, the blowfly larvae were concentrated in the head area and later moved to the abdomen after two days. As the decomposition progressed, the odour of decay intensified and more flies such as blowflies, flesh flies, and muscid flies arrived at the carcasses. Beetles remained present beneath the carcasses. The active decay stage lasted three days for CR carcasses but extended to five days for TR carcasses.

When third-instar larvae began leaving the carcasses to pupate, the decomposition entered the advanced decay stage. During this stage, most of the carcasses' original mass had been consumed, leaving behind skin, fur, and bones. Muscid flies were observed together with carrion beetles on the ground and underneath the CR carcasses. This stage lasted only 24–48 hours for CR carcasses. The carcasses then transitioned to the dry remains stage, marked by the desiccation and retention of bones.

On the other hand, the larvae were still actively feeding on the decomposed tissues, causing the TR carcasses to remain wet and thereby prolonging the active decay stage by four days. Fluid accumulation and tissue liquefaction, combined with the larvae's feeding activity as well as their secretions and excretions, contributed to the moist environment observed in the TR carcasses. On the 10th day, the TR carcasses began to dry, and decomposition was completed by the 12th day. The presence and absence of insects on rat carcasses of both groups were presented in Table 3.

Coumarin detection by GC-MS

In this study, the predominant fly larvae colonising both groups of rat carcasses were *C. rufifacies.* Therefore, 1 gram of the whole *C. rufifacies* larvae was used for the qualitative detection of secondary bioaccumulation of coumarin. The data was collected in the selected ion monitoring (SIM) technique where the molecule peak of coumarin was selected at m/z 146. The GC-MS analysis had shown that there is no trace of coumarin detected in CR samples. Meanwhile, in TR samples the coumarin molecular peak was detected at 15.25 minutes, which matched the molecule peak obtained from the National Institute of Standard Technology (NIST) database library (Figure 2).

Table 3. The insect colonization patterns associated with decomposition stages of rat carcasses in the control and coumarin-intoxicated carcasses group

Order	Family	Genus/Species	Decomposition stages									
			CR					TR				
			Fresh	Bloated	Active decay	Advanced decay	Dry	Fresh	Bloated	Active decay	Advanced decay	Dry
			Day-1	Day-2	Day-3-5	Day-6-7	Day-8	Day-1	Day-2	Day-3-7	Day-8-11	Day-12
Diptera	Calliphoridae	*C. rufifacies*	√	√	√	√			√	√	√	
		C. megacephala	√	√	√	√				√	√	
		H. ligurriens		√	√					√	√	
		Hy. violacea		√								
		Lucilia sp.		√								
	Sarcophagidae	*Sarcophaga* sp.		√						√	√	
	Muscidae	*Ophyra chalcogaster*			√	√				√	√	
		Atherigona orientalis				√				√	√	
Coleoptera	Scarabaeidae	*Onthophagous* sp.				√			√	√	√	
	Hydrophilidae	Unidentified				√			√	√	√	
	Staphylinidae	*Aleochara* sp.				√			√	√	√	

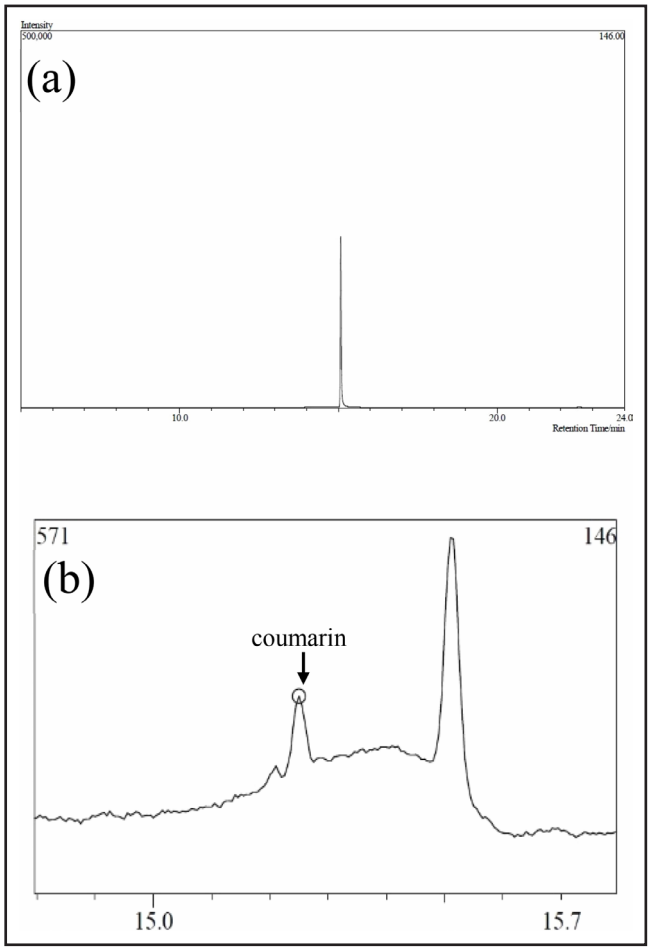

Figure 2. SIM chromatograms of coumarin selected at m/z 146 showing (a) Chromatograms of standard coumarin at 15.01 minutes, and (b) chromatograms of coumarin at 15.25 minutes extracted from *Chrysomya rufifacies* larvae infesting coumarin-intoxicated rat carcasses.

DISCUSSION

In the present study, five stages of decomposition which consist of fresh, bloated, active decay, advanced decay and dry remains were observed on carcasses both in the control and treatment group which similar to the previous works by Azwandi *et al*. (2013), Chen *et al*. (2014) and Silahuddin *et al*. (2015), to name a few. Other studies have determined the stages of decomposition into four (Ekanem & Dike, 2010; Mabika *et al*., 2014; Albushabaa & Almousawy, 2016) and six (Payne, 1965) stages. The decomposition duration for coumarin-treated carcasses was significantly prolonged, taking 12 days to reach the dry remains stage compared to eight days for control carcasses. The delay in decomposition corresponds with the delay in insect arrival and limited species of flies colonising the carcasses during the early stages of decomposition. These findings are consistent with previous research indicating that toxic substances can disrupt the typical succession of necrophagous insects, thereby altering decomposition dynamics (Zou *et al*., 2013; Rahim *et al*., 2024). For instance, pesticides like pirimiphos-methyl (Abd El-Bar & Sawaby, 2011), thiamethoxam (Eulalio *et al*., 2023), and chlorpyrifos (Rahim *et al*., 2024), among others, have been shown to delay the decomposition of animal carcasses.

Previous studies have demonstrated that a delay in insect arrival and reduced number of colonising insects can prolong the decomposition process. For example, the decomposition of rabbit carcasses intoxicated with pirimiphos-methyl was delayed by two to three days during the active decay stage and by approximately 21 days during the advanced decay stage (Abd El-Bar & Sawaby, 2011). Similarly, a study involving rat carcasses exposed to chlorpyrifos reported a six-day delay in the active decay stage compared to the control group (Rahim *et al*., 2024). These delays were attributed to the lack of insect colonization on the intoxicated carcasses, likely due to the masking of decay odours by the toxic substance (Voss *et al*., 2008). In the current study, insects' arrival was delayed by 24 hours. However, although the total number of insects was not reduced compared to the control carcasses, the intoxicated carcasses still took longer to decompose, particularly during the active and advanced decay stages.

Previous literature has discussed the insecticidal properties of coumarin (Chiasson *et al*., 2001; González-Coloma *et al*., 2005; Gupta & Misra, 2006; Prasad *et al*., 2006; Sharma *et al*., 2006) that might contribute prolong decomposition of the intoxicated carcasses. The low concentration of coumarin in the carcass tissues may not be lethal to the fly larvae consuming the tissues but may slows down the larvae development, thereby delaying the progression of the carcasses from decay to dry stage. In previous studies, several toxicants have been found to prolong the developmental rates of flies which include, pesticides (Mahat *et al*., 2009; Sari *et al*., 2023), drugs (Oliveira *et al*., 2009; Lü *et al*., 2014; Shamsuddin *et al*., 2018) and heavy metals (Moe *et al*., 2001; Raise & Gemmellaro, 2024). For example, lorazepam, has caused developmental delays in *C. rufifacies* by 350.72 hours compared to the control life cycle which took about 257.26 hours (Annasaheb & Ramrao, 2023). Similarly, exposure to malathion pesticides have been reported to delay the development of *Chrysomya megacepahala* larvae by 20 to 44 hours on malathion treated carcasses (Yan-Wei *et al*., 2010), and pupation period of *C. megacephala* by 48 to 72 hours (Mahat *et al*., 2009). These findings highlight the importance of considering delayed larval development on intoxicated carcasses when estimating the minimum postmortem interval (minPMI) in forensic cases involving toxic substances.

In a decomposition study involving warfarin-intoxicated carcasses, the findings were differed from those of the current study, even though warfarin is a derivative of coumarin. In their study, the decomposition duration was shorter in intoxicated carcass by two days compared to control carcasses (Abd El-Gawad *et al*., 2019). They discussed in detail that the rapid decomposition process was due to the compound, warfarin, did not mask the carcass's odour and thus did not alter insect activity (Abd El-Gawad *et al*., 2019).

Toxic compounds are known to impact insect colonization and development on contaminated carrion (Eulalio *et al*., 2023). In the current study, however, coumarin did not affect the colonisation of fauna on intoxicated carcasses, as flies from the forensically significant families Calliphoridae, Sarcophagidae, and Muscidae were observed and collected. Dipteran assemblage was similar in both groups of carcasses where the predominant flies colonising the carcasses was calliphorid flies, *C. rufifacies* and *C. megacephala* which have also reported previously in Malaysia (Maramat & Rahim, 2015; Silahuddin *et al*., 2015; Musa *et al*., 2024; Rahim *et al*., 2024). The arrival pattern differed for intoxicated carcasses, as only *C. rufifacies* arrived and oviposited during the bloated stage, whereas the control carcasses attracted a greater diversity of fly species during the early stages of decomposition. The arrival of other fly species, such as *Hemipyrellia ligurriens* and *Sarcophaga* sp., was delayed in the intoxicated carcasses, with their presence observed only during the active decay stage when the odour of decomposition became more intense. This is similar to the findings of Eulalio *et al*. (2023) where greater activity of insects was observed from the decay stage forward as there is skin disruption and decomposed fluid released on the soil (Oliveira-Costa *et al*., 2014). Other study which investigates the effect of malathion on fly succession and development (Yan-Wei *et al*., 2010) has observed the presence of *C. megacephala* on intoxicated carrion during early stages of decomposition. However, its immatures did not survive especially in carcasses that were treated with a higher dose of malathion (Yan-Wei *et al*., 2010).

In terms of insect abundance in control and treatment groups, the insects collected on treated carcasses were insignificantly higher compared to the control group. This may be due to the prolonged wet decomposition phase of intoxicated carcasses, during which decay odours persisted in the environment for a longer period of time, subsequently attracting more flies and other insects, such as beetles. A similar observation was made in pig carcasses treated with thiamethoxam (organophosphate), where a higher number of insects were collected on treated carcasses compared to control carcasses. The total decomposition time for treated pig carcasses was also longer compared to control carcasses (Eulalio *et al*., 2023).

Ambient temperatures, season and microclimate in postmortem environment also play an important role in decomposition rate and insect colonisation pattern (Sharanowski *et al*., 2008). In this study, there is a significant difference of temperature and relative humidity recorded among three trials of the experiment. However, there is no significant difference in insect abundance and succession pattern in both groups of carcasses between three trials of the experiment. Several studies have been conducted to observe and record carrion decomposition and insect activities in different seasons where there is a significant change in ambient temperatures and humidity (Sharanowski *et al*., 2008; Griffiths *et al*., 2020). The finding shows that pig carcass placed in summer (19.08°C) attracts more fauna than carcasses in spring (15.44°C) and fall season (9.36°C) (Sharanowski *et al*., 2008). Previous research in Australia on decomposition rate and insect succession pattern was carried out in different seasons also had shown that a faster decomposition rate was observed in the summer, where the ambient temperature was recorded up to 34°C and relative humidity was 56% (Griffiths *et al*., 2020). This environmental condition is optimal for the decomposition process both by bacteria and insect development (Griffiths *et al*., 2020).

As mentioned earlier, the presence of coumarin altered the rate of decomposition, likely due to the slower development of larvae ingesting the substance. The changes observed might be due to the coumarin bioaccumulation inside the fly larvae while feeding on the decomposing intoxicated carcasses. The bioaccumulation compound may interfere with the physiology of the larvae and can be detected through toxicological analysis (Sari *et al*., 2023). This is in agreement with previous literature (Carvalho *et al*., 2001; Mahmood *et al*., 2015) that mentioned necrophagous larvae can bioaccumulate drugs or poisons while feeding on decomposing bodies. However, the recoverability is dependent on the condition of the body of the necrophagous larva and the chemistry of drugs or poisons involved (Sari *et al*., 2023). In this study, qualitative detection carried out on third instar larvae of *C. rufifacies* had shown the presence of coumarin when analysed using Gas Chromatography-Mass Spectrometry (GC-MS). Third-instar larvae were used as the specimens because the peak concentration of the analyte can be detected during this stage due to its active feeding stage (Sadler *et al*., 1995). Positive detection of coumarin shows that *C. rufifacies* larvae were able to bioaccumulate in larvae tissues. Previous studies have shown that the bioaccumulation of toxins within larval tissues can alter the developmental patterns of necrophagous flies (e.g., Gennard, 2012; Rivers & Dahlem, 2014; Mahat *et al*., 2019).

The detection of toxins in entomological specimens is considered crucial as an alternative for toxicological analysis, particularly when suitable biological samples are unavailable due to advanced decomposition. The use of entomological specimens for toxicological diagnosis has been previously documented (Gennard, 2016; Magni *et al*., 2018; Mahat *et al*., 2019). Extensive research has been conducted on the effects of pesticides on entomological samples, given their impact on insect growth (Mahat *et al*., 2014; Denis *et al*., 2018; Abd Al Galil *et al*., 2021). However, such analyses have primarily focused on a limited range of pesticides, including organophosphates (Rashid *et al*., 2008; Liu *et al*., 2009; Yan-Wei *et al*., 2010; Dowling *et al*., 2022), the herbicide paraquat (Lawai *et al*., 2015), and aluminum phosphide (AlP) (El-Ashram *et al*., 2022).

Selecting appropriate extraction and instrumental techniques with highly sensitive and selective is important to ensure the toxins detection and confirm their involvement surrounding the death (Gosselin *et al*., 2011). In this study, QuEChERS extraction method was used as the extraction method to extract coumarin compound in fly larvae tissues. This method is rapid, simple and cost-effective (Wolstenholme *et al*., 2021). Previously, this method was used to extract toxin compounds (e.g., pesticides) in fruits and vegetables (Li *et al*., 2017). However, the method was revalidated by Magni *et*

al. (2018) to extract α- and β-endosulfan in *Calliphora vomitoria*. Besides pesticides, QuEChERS has also been utilised in extracting other analytes including therapeutic drugs and toxins from different matrices (Matsuta *et al*., 2013; Zhou *et al*., 2017). According to Sari *et al*. (2023), the use of QuEChERS in processing entomological samples is promising, therefore its application should be encouraged.

Analytical methods that usually used for analysing entomological specimens include; Radioimmunoassay (RIA), Gas Chromatography (GC), Gas Chromatography-Mass Spectrometry (GC-MS), Thin-Layer Chromatography (TLC) (Goff & Lord, 2001), High Performance Liquid Chromatography-Mass Spectrometry (HPLC/MS) (Heinrikson & Meredith, 1984; Goff & Lord, 2001), infrared spectroscopy (Barbosa *et al*., 2018), fluorescence analysis (Azab *et al*., 2015; Feng *et al*., 2018) and ultra violet (UV) visible spectroscopy (Bazrafshan *et al*., 2017; Ahmed *et al*., 2018) to name a few. In this study, detecting trace amount of coumarin in larvae samples using GC-MS was more suitable since coumarin is an aromatic organic compound (Das & Banik, 2020). GC-MS was employed for its ability to analyse volatile compound with high sensitivity and better identification ability with the presence of the NIST library database (Lynch, 2017).

Other compounds that were also detected by GC-MS include; α and β endosulfan (organochlorine insecticides) (Magni *et al*., 2018), paraquat (Lawai *et al*., 2015), nicotine (Magni *et al*., 2016) and metamphetamine (Magni *et al*., 2014). In the current study, the coumarin compound was detected using a single ion monitoring (SIM) technique at m/z 146. This method is commonly employed for trace analysis, where the mass spectrometer is programmed to specifically monitor the intensity of selected m/z values rather than scanning across a predefined mass range to acquire full mass spectra (Clench & Tetler, 2000).

The findings of this study highlight the effect of coumarin on the decomposition process and insect activity on intoxicated carcasses, demonstrating its potential to prolong the decomposition process. The positive detection of coumarin in larval tissues underscores the significance of entomotoxicological analysis in forensic investigations, offering valuable insights into the role of chemical substances in carrion decomposition within intoxicated environments.

CONCLUSION

The decomposition was delayed in coumarin-intoxicated rat carcasses. The duration of decomposition of rat carcasses in coumarin was longer which took about 12 days in relative to control carcasses which took about eight days to complete. The delay in the rate of decomposition of carcasses intoxicated with coumarin might be due to their insecticidal activity which could slows down the larvae activity in the decomposed carcasses. A higher number of insects were collected in the treated compared to the control group of carcasses, although not significant. However, the insect succession pattern of rat carcasses was slightly different compared to control carcasses. The toxicological analysis has shown that coumarin is bioaccumulated inside the *C. rufifacies* larvae tissues and the detection is possible with the utilization of GC-MS.

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Data availability Statement

The data for the findings of this study will be available upon request to any of this article's authors.

Conflict of Interest

The authors declare that they have no conflict of interest.

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