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Forensic Implications of Blowfly *Chrysomya rufifacies* (*Calliphoridae: Diptera*) Development Rates Affected by Ketum Extract

A. R. Rashid, A. S. Siti, F. R. Siti, A. R. Reena, H. S. S. Sharifah, F. Z. Nurul, and W. A. Nazni

***Abstract*—**This study was conducted to examine the effects of ketum extract on development of *Chrysomya rufifacies* and to analyze the presence of mitragynine in the larvae samples. 110 newly emerged first instar larvae of *C. rufifacies* were introduced on ketum extract-mixed cow liver at doses of 0, 20, 40 and 60g. Blowfly development rate was determined with 12 hour intervals and mitragynine in larvae was extracted and quantitated. *C. rufifacies* in control group took about 192 hours to complete their development from first instar larvae to adult blowfly; meanwhile blowfly form from the highest dose of ketum was 264 hours. Mitragynine was detected in all groups of treatment, except for control. In conclusion, the presence of mitragynine in *C. rufifacies* is affected in delaying development rates of the blowfly for up to 62 hours or 3 days. Chemical analysis of mitragynine from larvae samples showed that this alkaloid present in all specimens analyzed.

Keywords*—Chrysomya rufifacies*, Ketum, Mitragynine, Postmortem Interval (PMI).

I. Introduction

FORENSIC investigation usually deals with several main questions that need to be answered in order to clarify a death case without reasonable doubt. Death due to intoxication of drugs or poisons is one of the most prominent causes of death worldwide. In a dead remain, decomposition process occurred immediately after death and blowflies play an important role in this process as the main processor of the dead body. Entomologists were able to estimate the time of death by identifying the age of blowflies found on the corpse. This field, known as forensic entomology, applied the use of insects to aid in legal investigations [1]. The main aim of forensic entomology is to establish the postmortem interval (PMI) which is the time elapsed since death or, more exactly how long the dead body has been exposed to the environment [2]. Most of the invertebrate found on the dead body are *Diptera* (flies) and *Coleoptera* (beetles). Usually, these insects used the dead remain as a food source or nutrient, as well as to invade their eggs or hatch their larvae. Initially, these insects attract to the body fluid such as urine, saliva and fecal from the natural opening and blood from the wound. Later on, their immature consume the flesh and other tissues for living.

To apply insects in forensic investigations, the insect specimens such as blowfly larvae (maggots) and adults are considered as physical evidence, just like bloodstain, fingerprint, hair and fiber, or any biological materials [3]. Besides estimation of PMI, entomological evidence is also used to provide information on when, where, and how, under certain conditions, a crime was committed or a person died [4].

In addition, several studies have been conducted to investigate the influence of several factors such as nutrition, drugs and also type of organ involved as food source in blowfly growth phases [5]-[7]. The presence of drugs in corpse reported to affect the development of such insects since they ingested the drugs from corpse tissue into their system while feeding the remains [8]. This will give a huge impact in estimation of PMI since it is done using the age of blowfly larvae.

Ketum (*Mitragyna speciosa* Korth), also known as kratom is a *Rubiaceae* family member that exhibits a narcotic effect on human and this plant usually has been consumed as drink. There were over 25 alkaloids that has been isolated from ketum and the primary constituent of which is the indole alkaloid, mitragynine [9]. Its availability in black market and also in the net may increased the use of this plant to replace other narcotic drugs. Overdose intake of ketum leads to intoxication which can cause death [10]. Presence of the active alkaloid in this plant has been examined to delay the growth rates of blowfly *Chrysomya megacephala* and it affected the estimation of PMI using *C. megacephala* larvae up to 24 hours [11]. *C. megacephala*, along with *C. rufifacies* is the most abundant necrophagous blowflies found in Malaysia [12].

The objectives of the present study are to determine the effect of ketum extract on development rate of *Chrysomya rufifacies* and also to determine ability of *C. rufifacies* larvae in detection of mitragynine, in which both could be applied in exploring the PMI and cause of death in a real ketum intoxication death case.

II. Materials and Methods

A. Extraction of Mitragyna speciosa Korth

60g of dried and crushed *Mitragyna speciosa* Korth leaves were boiled in 1L of distilled water for 15min. Ketum extract obtained was poured through the strainer into the bowl.

A. R. Rumiza is with the Institute of Forensic Science, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, MALAYSIA (corresponding author phone: +603-55437855; fax: +603-55444565; e-mail: rumiza9550 @salam.uitm.edu.my).

A. S. Siti, F. R. Siti, A. R. Reena, H. S. S. Sharifah, F. Z. Nurul are with the Chemistry (Forensic Analysis) Programme, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia.

W. A. Nazni is with the Medical Entomology Department, Institute of Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia.

Leaves left on the strainer were once again boiled with 1L of distilled water for another 15min. The mixture then was strained, produced another extract. Finally, this extract was combined with the previous extract and boiled again until reached 1L of boiling extract. The boiled extract then was left to cool at room temperature.

B.Preparation of Ketum-Mixed Liver Samples

Cow livers were used as food substrate for blowfly larvae growth. Cow livers were homogenized with ketum water extract at different doses which were 0g, 20g, 40g and 60g. Each liver batch were mixed vigorously and soaked with 10mL of ketum water extract respectively for 4 hours. One group of the cow liver without ketum was used as a control.

C.Exposure of Chrysomya rufifacies Larvae to Ketum-Mixed Liver

Chrysomya rufifacies used in this study were obtained from Entomology Department, Institute of Medical Research (IMR), Kuala Lumpur, Malaysia. The first instar larvae of *C. rufifacies* were introduced into each doses of mitragynine. 110 newly emerged *C. rufifacies* eggs were introduced into each treatment. The larvae then were allowed to grow and complete their life cycle on the ketum-mixed liver at temperature 33°C. Observation on development of *C. rufifacies* larvae was done every 12 hours. Development rate was observed from larvae length, duration of each stage of development and survival rates of each stages of development in different doses of ketum, 0g, 20g 40g and 60g.

D.Development Rate of Larvae Feeding on Ketum-Mixed Liver

Development of larvae feeding on ketum-mixed liver was monitored starting from hatch and introduced into the liver until they formed adults. There were four rearing plastic boxes containing liver that was homogenized with different doses of ketum. A square hole (5cm x 5cm) were made on the lid of the boxes and covered with fine material for ventilation and to avoid contamination from small insects. Then, the rearing plastic boxes were put into an aquarium and the lid of the aquarium was also covered with the fine material. Along inside the aquarium, there were tissues put in as a dry area for pupation. The larvae then was left to grow until become pupae and adult.

During development in larvae stage, length of 10 randomly selected larvae of *C. rufifacies* from each treatment was measured every 12 hours until formed pupae. Duration for each stage of development and the survival rate also monitored. During post-feeding stage, 10 larvae were taken randomly out from each treatment and preserved in 70% ethanol for chemical analysis. The other larvae were let to grow until become adults.

E.Chemical Analysis of Chrysomya rufifacies Larvae Samples

Solid phase extraction (SPE) was used for extraction of mitragynine from larvae samples. Approximately, five (5) larvae from each dose were weighed and homogenized in 5mL phosphate buffer saline (PBS) by grinding the larvae samples using mortar and pestle. The homogenized larvae samples were then centrifuged at 3000rpm for 10min. Next, the supernatant was added with 8µL of 8mg/mL of 1, 4-diphenoxybenzene as internal standard. 1mL of the mixture then was loaded into Oasis HLB SPE cartridge [11].

The stationary phase of SPE cartridge was conditioned prior to sample loading with methanol. 5mL of sample were loaded into each cartridge at 48drops/min. The interference was then removed with distilled water. Finally, series of methanol: water composition (90:10, 50:50, 10:90) was loaded to elude mitragynine.

The presence of mitragynine in larvae samples were analyzed using the High Performance Liquid Chromatography (HPLC) with the flow rate of 1.0mL/min and the detector was set at 223nm. Mobile phase used was acetonitrile and water in different ratio (90:10). The injection volume was 1µL. Analytical column used was C18 (250mmx4.6mm, 5µm) with guard column C18 (4.6mm, 5µm).

F.Mitragynine Calibration Curve

A series of mitragynine standard concentration were prepared to obtain the calibration curve. 1000µg/mL stock of mitragynine standard was diluted to prepare the standard solution of mitragynine which were 0.01µg/mL, 0.05µg/mL and 0.1µg/mL.

G.Statistical Analysis

All the data obtained from the study were subjected to statistical analysis to compare the differences between the control and the test groups. Statistical analysis was done using ANOVA test.

III.Results and Discussions

A.Larvae Length of Chrysomya rufifacies

Fig. 1 represented the mean of larvae length collected randomly every 12 hours. Larvae length from all different doses did not show a significant difference at the early time of exposure, especially during the first 12 hours. Development of *C. rufifacies* larvae exposed to different doses of ketum extract was found to be slightly slower particularly during 24 to 60 hours of development, if compared to control larvae. From statistical analysis, there were no significant differences in larvae length from control group compared to 20g, 40g and 60g of ketum groups between 24 hours to 60 hours of exposure, with p value>0.05. However, at 60 hours, larvae length of control and 60g groups was found to have a significant difference with p= 0.028.

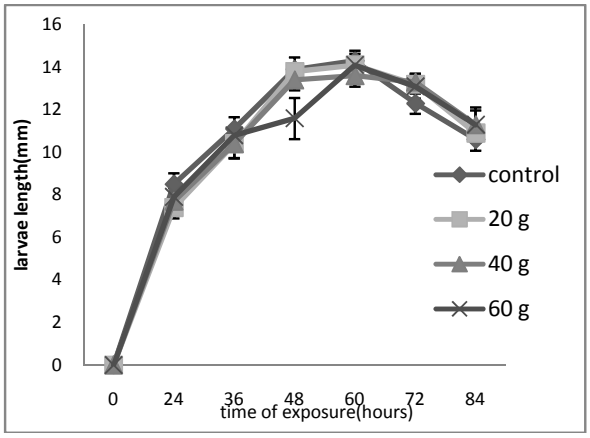

Fig. 1 represents Mean of larvae length (±SD) (mm) of *Chrysomya rufifacies* consumed cow liver containing different concentrations of mitragynine

During the next 72 hours until 84 hours of exposure, there were significant differences in all groups of treatment if compared to control (p<0.05). At this time, larvae length started to decrease because of most larvae went through the post-feeding stage, whereby they were wandering away from food source and prepare to pupate. These may explain the shortened in their length during this moment.

The differences in larvae length after 72 hours suggested that the application of larvae in estimation of PMI in death case due to ketum intoxication after 72 hours of death is invalid if it is being done by comparing larvae age found on corpse with the normal life cycle table of *C. rufifacies*. The differences on duration of each stages of development have to be considered on larvae specimens which contained ketum or mitragynine.

The result obtained from this study was similar with previous studies done in our laboratory, also using ketum [12] and malathion [13] on blowfly *Chrysomya megacephala*. Both reports showed that the presence of ketum and malathion in larvae system of *C. megacephala* significantly delayed the development of this blowfly species, based on the larvae length. Somehow, there were also studies reported the acceleration of blowfly development caused by some drugs such as paracetamol [14] and diazepam [15] on different species of blowflies. In addition, some drug such as morphine [16] gives no effect on *Calliphora stgyia* larvae development. It was found that the rates of development of blowflies were different between species and the presence of different drugs in system also gave different effects in blowfly development.

B.Survival Rate of Chrysomya rufifacies Larvae

TABLE I
SURVIVAL RATES (%) OF BLOWFLY *CHRYSOMYA RUFIFACIES* BASED ON DIFFERENT DOSES OF KETUM

Ketum doses (g)	Stages of development		
	Larvae	Pupae	Adult
Control	97	94	43
20	68	68	13
40	87	86	21
60	81	81	43

Based on data obtained in Table I, percent of control larvae survived and pupae survived were higher compared to *C. rufifacies* exposed to ketum extract. Survival rate of *C. rufifacies* recorded was decrease in increment of ketum extract doses. However, adult emerged from 60g of ketum treatment was higher compared to the other two lower doses on 20g and 40g of treatment. During larvae stages, these immature blowflies were undernourished and this leads to lower number of pupae that survived to form adult. For 20 g treatment, it showed lower survival rate for adults which were 13 %.

C. Duration of Development of Chrysomya rufifacies Larvae

The fully mature third instar of *C. rufifacies* was robust and mucoid-shaped with the length of ≈14mm [7]. Usually, development of *C. rufifacies* is also affected by the temperature. In this study, temperature used was constant at 33°C. Usually blowflies grow about 2.5mm to 4mm (first instar), shed its skin and grow about 5mm to 8mm (second instar) and shed its skin again and grow to about 15mm to 22mm (third instar) [17].

TABLE II
TOTAL DEVELOPMENTAL DURATION (HOURS) OF *CHRYSOMYA RUFIFACIES* IN EACH STAGES OF LIFE CYCLE

Stages of development		Ketum doses (g)			
		Control	20	40	60
Larvae	1st instar	x ≤12	x ≤9	x ≤6	x ≤6
	2nd instar	12 ≤x≤ 21	9 ≤x≤ 18	6 ≤x≤ 24	6 ≤x≤ 30
	3rd instar	21 ≤x≤ 63	21 ≤x≤ 63	64 ≤x≤ 72	30 ≤x≤ 54
Prepupae		63 ≤x≤ 87	60 ≤x≤ 87	72 ≤x≤ 93	54 ≤x≤ 99
Pupae		87 ≤x≤ 192	87 ≤x≤ 200	93 ≤x≤ 247	99 ≤x≤ 264

Based on data obtained in Table II, duration of development of *C. rufifacies* at 40g and 60g were slightly similar. Development from larvae to pupae of the highest doses was delayed up to 12 hours if compared to control. It took about 4.1 days (±99 hours) from larvae to become pupae, compared to 87 hours (3.6 days) in control. *C. rufifacies* exposed to 20g of ketum extract showed development rate which are quite similar with control from first instar until it formed pupae. Both took 87 hours to develop from first instar larvae to pupae.

In overall, the growth of *C. rufifacies* on the highest concentration of mitragynine were delayed in 3 days with the total development period was 11 days (264 hours) compared with control which only takes about 8 days (192 hours) to

complete the cycle from larvae to adults. If larvae samples are used for estimation of PMI, the presence of mitragynine in the samples has to be taken into consideration, since it has been shown that ketum delayed the development of *C. rufifacies* up to 3 days.

Previous developmental study done using ketum on *C. megacephala* also showed the delay in this blowfly growth for 24 hours compared to control [11]. The same findings also reported a delay in blowfly *C. megacephala* exposed to the malathion in which the total development time was extended to 10 days rather than 7 days in control [13].

D.Qualitative Analysis of Mitragynine from Chrysomya rufifacies Larvae Using High Performance Liquid Chromatography (HPLC)

Fig. 2 showed the calibration standard of mitragynine obtained from the concentration of 0.01µg/mL, 0.05µg/mL and 0.1µg/mL. This graph was plotted in order to estimate the concentration of mitragynine recovered from the larvae sample.

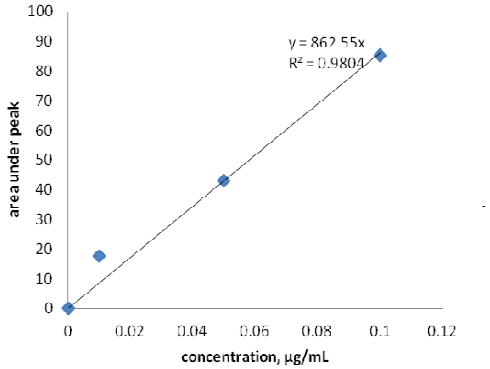

Fig. 2 represents Standard calibration curve of standard mitragynine (µg/mL)

From the data analysis done using the HPLC, most of mitragynine peak appear at the retention time ± 2.2 minutes at different concentrations (Fig. 3). However, there was no peak for control appears due to the absence of mitragynine in the larvae sample. From calibration curve, the concentration of mitragynine in larvae samples at different doses which 20g, 40g and 60g were calculated. Based on the calibration curve, standard deviation of the response and the slope, the LOD (limit of detection) and LOQ (Limit of quantitation) obtained were LOD = 0.000857 and LOQ = 0.0286.

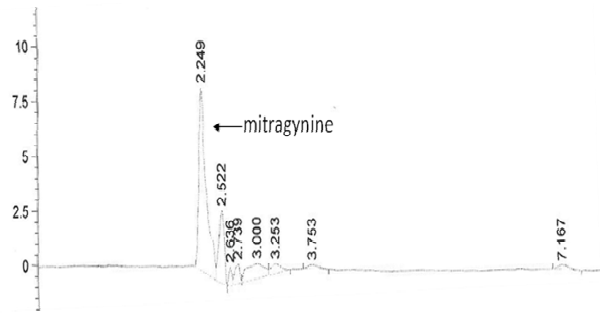

Fig. 3 Chromatogram of mitragynine from *C. rufifacies* larvae samples from 20g ketum

TABLE III
CONCENTRATION OF MITRAGYNINE (µG/ML) IN LARVAE SAMPLE FROM DIFFERENT DOSES OF KETUM EXTRACT

Ketum extract dose (g)	Concentration of mitragynine
20	0.167
40	0.324
60	0.650

Table III showed different concentrations of mitragynine recovered from larvae samples. Concentration of mitragynine in larvae exposed to the highest dose of ketum extract was detected as the highest if compared to other two doses. It seems that with the increment of ketum extract exposed, the concentration of mitragynine was also increased simultaneously.

If larvae feed on tissue contained some kind of drugs or substances, there were two processes that might happen which were bioaccumulation, or an excretion of the drug and its metabolites. The present report deals with the effect and detection of mitragynine on blowfly tissue and we could verify that there were bioaccumulations since the presence of drug had significant impact on larvae growth, puparation and adults emergence.

IV. CONCLUSION

In conclusion, different doses of ketum consumed by larvae affected in delaying development rate of blowfly of *Chrysomya rufifacies* for up to 3 days. Mitragynine, the most active constituent in ketum, was able to be detected in *C. rufifacies* larvae samples at different concentration and this suggested that *C. rufifacies* larvae sample could be used as alternative specimen in intoxication cases of ketum.

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