INSECTICIDAL AND REPELLENT PROPERTY OF SELECTED ZINGIBERACEAE SPECIES AGAINST MEDICALLY IMPORTANT MOSQUITOES

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ABSTRACT

Mosquitoes are the vectors for transmitting severe and well-known illnesses such as malaria, yellow fever, dengue fever, chikungunya, filariasis, encephalitis, West Nile virus and Zika virus. These diseases produce significant morbidity and mortality in human worldwide. Of late the utilisation of environment friendly and biodegradable natural insecticides of plant origin are preferred because synthetic insecticides not only cause development of resistance in vector species but are also harmful to man and environment. Hence, this study explored the effects of hexane and dichloromethane extracts of *Zingiber officinale* var. *rubrum* (HZOR and DZOR), *Zingiber montanum* (HZM and DZM), *Zingiber spectabile* (HZS and DZS), *Zingiber zerumbet* (HZZ and DZZ) and *Curcuma aeruginosa* (HCA and DCA) on larvicidal, adulticidal and repellent activities against *Aedes albopictus*, *Aedes aegypti* and *Culex quinquefasciatus*. The hexane and dichloromethane extracts were prepared by soaking the rhizome powder into two organic solvents, hexane and dichloromethane separately and then were filtered and evaporated. The yield obtained from hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* were 3.29%, 6.97%, 3.09%, 9.44% and 9.09%, respectively. The larvicidal and adult mortality were observed after 24 h of exposure; no mortality was observed in the control group. Results of log-probit analysis (at 95% confidence level) revealed that HZS, HZOR and HZM were noted to be active against the larvae of *Ae. albopictus* (LC_{50}= 93.51, 96.86, 99.04 mg/L; LC_{90}= 168.65, 168.65, 153.77 mg/L, respectively). The HZZ and HZM were recorded to be active against larvae of *Ae. aegypti* (LC_{50}= 82.05, 84.95 mg/L; LC_{90}= 121.05, 134.85 mg/L, respectively) whereas, HZZ, DZZ, HCA and DCA were noted to be highly active against larvae of *Cx. quinquefasciatus* (LC_{50}= 49.28, 30.15, 21.94, 42.47mg/L; LC_{90}= 83.87, 82.62, 66.61, 99.05 mg/L, respectively). The highest adult mortality was only

observed against *Ae*. *albopictus* mosquito and was found in HCA, HZS, HZZ and DZS with 37.78%, 24.44% 20.00% and 15.56%, respectively. Of the five Zingiberaceae species tested for repellent activity against the three mosquitoes at 1,000 mg/m^2, HZM and DZM were the most effective with 89.33% and 85.33% repellency against *Ae*. *aegypti* mosquito. Therefore, these results suggest that hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* have the potential to be developed as bio-insecticides to control the larvae, adult and as repellent agents for *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus*.

ABSTRAK

Nyamuk dikenali sebagai vektor utama yang membawa penyakit-penyakit bawaan vektor seperti malaria, demam kuning, demam denggi, chikungunya, filariasis, ensefalitis, jangkitan virus Nil Barat dan Zika virus. Penyakit tersebut telah menyebabkan kesan morbiditi dan kematian pada manusia di seluruh dunia. Sehingga hari ini, penggunaan racun serangga yang semula jadi dan mesra alam yang berasal daripada sumber tumbuhan lebih dipilih kerana racun serangga komersial bukan sahaja menyebabkan kerintangan dalam spesies vektor juga memberikan kesan buruk ke atas alam sekitar dan manusia. Kajian ini telah dijalankan untuk mengkaji keberkesanan ekstrak heksana dan diklorometana daripada rizom *Zingiber officinale* var. *rubrum* (HZOR dan DZOR), *Zingiber montanum* (HZM dan DZM), *Zingiber spectabile* (HZS dan DZS), *Zingiber zerumbet* (HZZ dan DZZ) dan *Curcuma aeruginosa* (HCA dan DCA) untuk aktiviti larvisid, aktiviti adultisid dan aktiviti penghalau, terhadap nyamuk *Aedes albopictus*, *Aedes aegypti* dan *Culex quinquefasciatus*. Ekstrak heksana dan diklorometana disediakan dengan cara merendam rizom tumbuhan yang telah dikisar halus di dalam kedua-dua pelarut organik tersebut secara berasingan dan seterusnya ditapis dan dikeringkan. Hasil yang diperolehi daripada larutan heksana dan diklorometana *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* dan *C. aeruginosa* adalah masing-masing sebanyak 3.29%, 6.97%, 3.09%, 9.44% dan 9.09%. Aktiviti larvisid dan adultisid diperhatikan selepas 24 jam pendedahan dan tiada kematian dilaporkan dalam kumpulan kawalan. Keputusan analisis log-probit (pada 95% tahap keyakinan) menunjukkan bahawa HZS, HZOR dan HZM adalah ekstrak yang mempunyai kesan aktif terhadap larva *Ae. albopictus* dengan nilai LC_{50} (93.51, 96.86, 99.04 mg/L) dan LC_{90} (168.65, 168.65, 153.77 mg/L) bagi tiap-tiap ekstrak tumbuhan. Ekstrak HZZ dan HZM telah direkodkan sebagai ekstrak yang aktif terhadap larva *Ae. aegypti* (LC_{50} = 82.05, 84.95 mg/L; LC_{90} = 121.05, 134.85 mg/L) manakala, ekstrak HZZ, DZZ, HCA dan DCA

adalah ekstrak yang mempunyai kesan sangat aktif terhadap larva *Cx*. *quinquefasciatus* (LC_{50} = 49.28, 30.15, 21.94, 42.47mg/L; LC_{90} = 83.87, 82.62, 66.61, 99.05 mg/L). Jumlah kematian nyamuk yang paling tinggi hanya dilihat pada *Ae*. *albopictus* dan ditemui dalam ekstrak HCA, HZS, HZZ dan DZS dengan peratusan 37.78%, 24.44%, 20.00% dan 15.56% masing-masing. Hasil daripada ujian aktiviti penghalau bagi lima species Zingiberaceae ke atas 3 nyamuk pada kepekatan 1,000 mg/m^2 mendapati ekstrak HZM dan DZM menunjukkan aktiviti yang paling efektif dengan 89.33% dan 85.33% terhadap nyamuk *Ae*. *aegypti*. Oleh yang demikian, keputusan ini menunjukkan bahawa ekstrak heksana dan diklorometana *Z*. *officinale* var. *rubrum*, *Z*. *montanum*, *Z*. *spectabile*, *Z*. *zerumbet* dan *C*. *aeruginosa* berpotensi untuk dikomersialisasikan sebagai bio-insektisid serangga untuk aktiviti larvisid, adultisid dan penghalau dalam mengawal nyamuk *Ae*. *albopictus*, *Ae*. *aegypti* dan *Cx*. *quinquefasciatus*.

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Ae. aegypti	:	*Aedes aegypti*
Ae. albopictus	:	*Aedes albopictus*
C/min	:	celcius per minute
cm	:	centimeter
cm^2	:	centimeter square
Cx. quinquefasciatus	:	*Culex quinquefasciatus*
C. aeruginosa	:	*Curcuma aeruginosa*
^oC	:	degree Celcius
DZM	:	dichloromethane extract of *Zingiber montanum*
DZOR	:	dichloromethane extract of *Zingiber officinale* var. *rubrum*
DZS	:	dichloromethane extract of *Zingiber spectabile*
DZZ	:	dichloromethane extract of *Zingiber zerumbet*
eV	:	electron Volt
g	:	gram
HCA	:	hexane extract of *Curcuma aeruginosa*
HZM	:	hexane extract of *Zingiber montanum*
HZOR	:	hexane extract of *Zingiber officinale* var. *rubrum*
HZS	:	hexane extract of *Zingiber spectabile*
HZZ	:	hexane extract of *Zingiber zerumbet*
h	:	hour
kg	:	kilogram
KD	:	knockdown
LC	:	lethal concentration
LT	:	lethal time
L:D	:	light : dark

L	:	litre
m	:	meter
µl	:	microlite
mg/L	:	milligram per litre
mg/m^2	:	milligram per metre square
ml	:	mililitre
mm	:	milimeter
min	:	minute
%	:	percentage
RH	:	relative humidity
Z. montanum	:	*Zingiber montanum*
Z. officinale var. rubrum	:	*Zingiber officinale* variety *rubrum*
Z. spectabile	:	*Zingiber spectabile*
Z. zerumbet	:	*Zingiber zerumbet*

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND OF STUDY

In tropical countries, insect vectors especially mosquitoes, contribute to a larger proportion of human health problems. They are responsible for spreading dangerous diseases such as malaria, dengue fever, yellow fever, filariasis, zika virus and other viral infections (Prajapati *et al*., 2005; Pushpanathan *et al*., 2008; Mlakar *et al*., 2016). Since the rapid industrial and economic development have brought new changes of infrastructure, higher number of mosquito breeding places have been created (Chua *et al*., 2005). It has also contributed to the increasing number of these diseases in today's society.

In Malaysia, *Aedes albopictus*, *Aedes aegypti* and *Culex quinquefasciatus* are three medically important vectors. *Aedes* species are known as vectors transmitting dengue and chikungunya virus, while *Cx. quinquefasciatus* species is a vector that transmits lympathic filariasis and Japanese encephalitis (Vinayachandra *et al*., 2011; Murugan *et al*., 2012; Vythilingam *et al*., 1995).

Dengue is becoming a major problem in most of the tropical countries. A study conducted by Brady *et al*. (2012) on the prevalence of dengue, currently estimates that 3.9 billion people, in 128 countries, are at risk of infection with dengue viruses (WHO, 2016). In Malaysia, the number of dengue cases has increased significantly in recent years. In the first national outbreak in 1973, 969 cases were reported; while in the next epidemic in 1982, 3005 cases were notified (Smith, 1956; Abubakar & Shafee., 2002). According to the latest data from Ministry of Health Malaysia (MOH) in 2017, the total

cases of dengue fever for the week 52 (25^{th}-31^{st} Dec 2016) was 1,329 cases and in week 01 (01^{st}-07^{th} Jan 2017), the number has slightly increased to 1,663 cases.

Lymphatic filariasis affects 120 million people worldwide and approximately 66% of those at risk live in the South-East Asia Pacific Region and 33% in the African Region (Noordin, 2007). In Malaysia, lymphatic filariasis was firstly observed in 1908 and transmitted by Malaysian *Cx. quinquefasciatus* mosquito (Vythilingam *et al*., 1995). Endemic cases were recorded from several states of Peninsular Malaysia, such as Terengganu, Kelantan, Pahang, Selangor and Johor as well as Sabah and Sarawak (Al-Abd *et al*., 2014; Noordin, 2007).

To date, the chemical insecticides remain as the main control agents against mosquito vectors. For example: the application of organophosphates like temephos (Abate) and fenthion to control the mosquito larvae; the ultra-low volume (ULV) fogging, thermal fogging, surface residual spraying and numerous household insecticide products were used to control the adult mosquitoes; or the usage of mosquito-repellent, such as DEET (N, N-diethyl-3-methylbenzamide) to prevent mosquito bite (Warikoo *et al*., 2012; Yang *et al*., 2002; Lee, 1997; Yap *et al*., 2002). However, the use of those insecticides in the long term have resulted in disruption of its natural biological control systems, increase the development of resistance, undesirable effects on non-target organisms, fostered environmental and human health concern (Govindarajan, 2010; Rahuman *et al*., 2008; Prajapati *et al*., 2005; Pushpanathan *et al*., 2008). Thus, a number of rules and regulations have been issued under the Environmental Protection Act in 1969 to check the application of chemical control agents in nature (Ghosh *et al*., 2012). These factors have highlighted the need for the development of new strategies in controlling mosquito population which is environmentally safe, cost-effective, and biodegradable.

Plants which have a rich source of bioactive compounds, can be developed as an alternative source of environmentally safe materials for mosquito larval control and pest-managing agents (Yang *et al*., 2004; Warikoo *et al*., 2012). According to Warikoo *et al*. (2012), the crude extracts from plant leaves, roots, seeds, flowers and barks have been used as insecticides for centuries. The insecticides derived from plant comprise botanical blends of chemical compounds, can be acted concertedly on both behavioural and physiological processes, differ from the conventional insecticides which are based on a single active ingredient (Ghosh *et al*., 2012). Botanicals obtained from plants resources are called phytochemicals which are naturally occurring insecticides. Several groups of phytochemicals such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported previously for their insecticidal activities (Shaalan *et al*., 2005). Roark (1947) described approximately 1,200 plant species having potential insecticidal value, while Sukumar *et al*. (1991) listed and discussed 344 plant species that only exhibited mosquitocidal activity.

Insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varieties and parts used, but also due to extraction methodology adopted and the polarity of the solvents used during extraction (Ghosh *et al*., 2012). Therefore, much effort has been focused on plant extracts or phytochemicals as potential sources of commercial mosquito control agents for the interruption of the transmission of mosquito-borne diseases at the individual as well as at the community level (Yang *et al*., 2004; Bagavan *et al*. 2008).

1.1 OBJECTIVES OF THIS STUDY

The purpose of this study is to determine the toxicity status of rhizome extracts of *Z. officinale* var. *rubrum, Z. montanum, Z. spectabile, Z. zerumbet* and *C. aeruginosa* against the larvae and adults of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*. The outcome of this study is essential in evaluating the potential of these plants to control the populations of larvae and adults of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*. The objectives are:

1. To evaluate the insecticidal activity of the hexane and dichloromethane rhizome extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*.
2. To determine the effective concentrations of larvicidal and adulticidal activity of the hexane and dichloromethane rhizome extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*.
3. To investigate the repellence, feeding inhibition and mortality effect of the hexane and dichloromethane rhizome extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*.

CHAPTER 2: LITERATURE REVIEW

2.1 The family Zingiberaceae

Zingiberaceae is the largest family in the order Zingiberales with 53 genera and over 1500 species that are spread mainly in the tropics from India to Malaysia (Larsen *et al*., 1999; Saensouk *et al*., 2015). Zingiberaceae species has been cultivated in India, China and Southeast Asian countries (Bua-in & Paisooksantivatana, 2009; Nampoothiri *et al*., 2012). The greatest concentration of genera and species is in the Malesian region (Indonesia, Malaysia, Singapore, Brunei, the Philippines and Papua New Guinea) (Larsen *et al*., 1999; Sirirugsa, 1998). Of the 53 genera and 1500 species known in the world, at least 20 genera and 300 species are found in Malaysia (Larsen *et al*., 1999; Kress *et al*., 2002; Holttum, 1950).

According to the latest classification system for Zingiberaceae, the family is divided into 4 subfamilies namely: Alpinioideae, Siphonochiloideae, Tamijioideae and Zingiberoideae; and 6 tribes namely: Alpinieae, Riedelieae, Siphonochileae, Tamijieae, Zingibereae and Globbeae (Kress *et al*., 2002). The species used in this study are cultivated species belonging to the subfamily of Zingiberoideae and tribe of Zingibereae.

Zingiberaceae species grow naturally in damp, shaded parts of the lowland or mostly humid shady places of the lowland or on hill slopes, as scattered plants or thickets; some are found infrequently in secondary forest; and some species can be fully exposed to the sun (Larsen *et al*., 1999; Sirirugsa, 1998; Habsah, *et al*., 2000).

2.2 The species used in this study

In this present study, four species from the genus *Zingiber* and one species from the genus *Curcuma* were studied for their insecticidal and repellent potential. The five species investigated are *Zingiber officinale* var. *rubrum* Theilade, *Zingiber montanum* (Koenig)

Link ex Dietr., *Zingiber spectabile* Griff, *Zingiber zerumbet* Smith and *Curcuma aeruginosa* Roxb. Below are the descriptions on the botanical aspect of each species.

2.2.1 *Zingiber officinale* var. *rubrum* Theilade

Botanical name : *Zingiber officinale* var. *rubrum* Theilade

Local name : Halia bara (Larsen *et al*., 1999; Holttum, 1950)

Figure 2.1: The rhizome of *Zingiber officinale* var. *rubrum* (Photo by: Prof. Halijah Ibrahim)

General description : It is cultivated in Southeast Asia mainly for medicinal purposes. This variety is morphologically similar to the common ginger (*Zingiber officinale*), but the rhizomes of this variant are smaller and have a stronger and more pungent smell (Ibrahim *et al*., 2008). The rhizome is red on the outside but is yellowish pink in cross section, coloured red at the base of the leaf shoot (0.5 m to 1.25 m in length) and has larger leaves and labellum (Theilade, 1996; Ibrahim *et al*., 2008). The petiole is also reddish when young with 2 mm long and the lip is scarlet red mottled with cream (Ibrahim *et al*., 2008). The spike is elliptic or oblong with 4-5 cm long. The bracteole is elliptic and often longer than the bract. The flower has 1.2 cm long calyx and 5 cm long yellow corolla. The labellum is scarlet red mottled with cream, 1.5 cm long. The flower also has cream anthers, dark purple appendages, red capsule and globose, sculpturing cerebroid pollens (Theilade, 1996).

2.2.2 *Zingiber montanum* (Koenig) Link ex Dietr. (Syn: *Zingiber cassumunar* Roxb. & *Zingiber purpureum* Roscoe)

Botanical name : *Zingiber montanum* (Koenig) Link ex Dietr.

Local name : Bonglai (Hamirah *et al*., 2010)

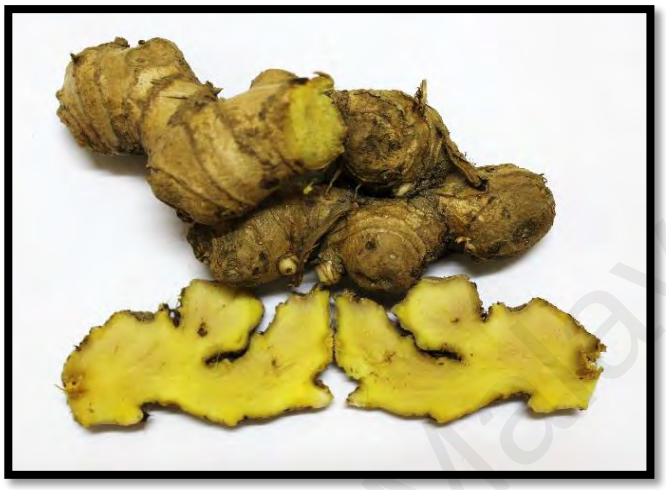


Figure 2.2: The rhizome of *Zingiber montanum*

General description : This species occurs widely as a home-garden plant in Southeast Asia. *Z. montanum* is a perennial, clumping herb. The rhizomes are horizontal creeping, tuberous, cylindrical to ovoid, irregular, palmately and profusely branched, laterally compressed and strongly aromatic with yellow flesh colour. Pseudostem is cylindrical, erect, enveloped by leafy sheaths and reaching 1.2-1.8 m high. Leaves are alternate, distichous, simple, subsessile or shortly petiolate, lanceolate-oblong and 3.5-5.5 cm by 18-35 cm long. Leaf sheaths are oblong, with membranous margins; ligules are ovate and membranous. Inflorescence is radical; spikes are cylindrical, fusiform or cone like, borne on a peduncle spike (scape) arising from rhizome and 8-60 cm high with 5-7 cataphylls; bracts are divided into outer and inner, spirally arranged, very dense, persistent and red or purplish brown; the outer is broadly ovate to suborbicular and cucullate, while the inner is ovate and glabrous. Flowers are ebracteolate, bisexual, zygomorphic and epigynous; calyx is 1.2-1.5 cm, membranous, glabrous and white; corolla has 4 lateral lobes and is linear-lanceolate, yellowish white and reddish lineolate on margins; labellum

is white or pale yellow, all in 2-3 cm long and 1.8-2.5 cm wide. The midlobe almost round, retuse at the apex when newly expanded and deeply split when old while the staminodes are much smaller (Lim, 2016; Holttum, 1950).

2.2.3 *Zingiber spectabile* Griff

Botanical name : *Zingiber spectabile* Griff

Local name : Tepus tanah and Tepus anjing (Larsen *et al*., 1999; Sivasothy *et al*., 2012)

Figure 2.3: The *Zingiber spectabile* species (left: the bract, right: rhizome)

General description : This species is native in the moist lowland forests of Peninsular Malaysia and found throughout Peninsular Malaysia. The leaves are large about 6-10 cm, glabrous or slightly hairy at the base beneath. No petiole. Inflorescence is about 12-30 cm long, cylindrical, not tapering to the apex and only a few flowers are produced by inflorescence at any one time. When young, the bract are yellow and it turn red when old. A single short-lived flower with pale yellow petals and a purple lip arises from the axil of each bract. The bracteole is about 4 cm long, split to the base and very short. The ovary is hairy and about 5 mm long. The length of calyx is about 2.7-3 cm. The corolla-tube is pale yellow and about 3 cm long. The labellum is approximately 2.5 mm long and the staminodes are erect on either side of the stamen (Ibrahim *et al*., 2008).

2.2.4 *Zingiber zerumbet* Smith

Botanical name : *Zingiber zerumbet* Smith

Local name : Lempoyang (Ibrahim *et al*., 2008; Kader *et al*., 2011)

Figure 2.4: Rhizome of *Zingiber zerumbet*

General description : This species widely cultivated throughout the tropics including Southeast Asia, Bangladesh, India, and Korea for its medicinal properties (Kader *et al*., 2011). The rhizome is perennial, thick, scaly, aromatic and pale yellow internally. The stems approximately 1-2 m tall, erect, oblique and round. The leaves, which are sometimes purplish beneath young shoots, are thin approximately 25-35 cm long. The petiole is about 6 mm long while the ligule, which is very thin, entire and broad, is approximately 1.5-2.5 cm long. The leaflets are arranged alternately. The inflorescence, which is approximately 6-12 cm long and green when young and becomes red when old, is borne on a separate pseudostem from the leaves and has closely overlapping bracts that form an open pouch in which flowers occur, one in each bract. It is a spike, ovoid to ellipsoid in shape; bracts subtend the position of each of the flowers giving the inflorescence its pinecone shape. The bracts are approximately 3-3.5 cm long and 2.5 cm wide while the bracteole is approximately 2.5 cm long, wide and thin but persistent until fruiting. The pale yellow or white flowers emerge from the lowest bract first, and when

exhausted, the flower dried and falls away. After flowering, the bracts change colour until the entire inflorescence is bright crimson. The corolla tube is as long as the bract while the style is long and filiform. The stigma is slightly projecting and its margin is ciliate. The stamen is attached with a long curved beak or horn-like shape. One stamen of the inner whorl is fertile, and the two staminoids have petal-like shape. The ovary is inferior and trilocular with axile placentation (Yob *et al*., 2011; Ibrahim *et al*., 2008).

2.2.5 *Curcuma aeruginosa* Roxb

Botanical name : *Curcuma aeruginosa* Roxb

Local name : Temu hitam (Ibrahim *et al*., 2008; Sirat *et al*., 1998)

Figure 2.5: Rhizome of *Curcuma aeruginosa*

General description : This species is native to Southeast Asia, including Myanmar, Cambodia, Vietnam, Thailand, Indonesia and Malaysia (Thaina *et al*., 2009; Suphrom *et al*., 2012). The rhizome of *C. aeruginosa* is about 16 cm long and 3 cm thick. The outside of the rhizome is grey and the tips is pink, but the inside is bluish or blue-green with white cortex. There is a purplish patch along either side of the midrib on upper side of the leaves which has the typical burgundy mid-stripe. Leaf sheaths are up to 50 cm long while leaf blades are about 30-80 cm x 9-20 cm, elliptical to oblong-lanceolate and green with purplish-brown. The inflorescence develops from the rhizome, usually

before the leaves are produced. The bracts are pale green and red-purple coma bracts. The flowers are 2-7 in axils of secondary bracts and the calyx is half as long as the corolla tube. The corolla is deep crimson-pink or red in colour, tubular at base, glabrous and about 4.5 cm long. The labellum is about 17 mm x 17 mm. The staminodes are pale yellow and longitudinally folded while the anther is spurred (Lim, 2016).

2.3 MEDICINAL IMPORTANCE OF SELECTED ZINGIBERACEAE SPECIES

Species from the family Zingiberaceae are known for their medicinal importance since ancient time, for example: *Zingiber officinale* Roscoe which has been used as a spice for over 200 years (Stoilova *et al*., 2007). *Zingiber zerumbet* rhizomes are also used in the traditional medicine in Asian, Indian, Chinese and Arabic folkfore since ancient time and have been consumed to cure swelling, loss of appetite, lumbago, diabetes, inflammation, chest pain, rheumatic pains, bronchitis, dyspepsia and sore throat (Kader *et al*., 2011; Yob *et al*., 2011).

In Japan, leaves of *Alpinia zerumbet* are sold as herbal tea and are used to flavor noodles and wrap rice cakes (Chan *et al*., 2009). The rhizomes of *Alpinia conchigera* are consumed as a port-partum medicine and the young shoots are prepared into a vegetable dish in some states of Peninsular Malaysia (Ibrahim *et al*., 2007; Saha & Paul, 2012).

The leaves and rhizomes of *Zingiber spectabile* are used as food flavouring, the pounded leaves are applied as a poultice to inflamed eyes and on to the body to reduce swelling (Sivasothy *et al*., 2013). Thaina *et al*. (2009) also reported that the rhizome of *Z. spectabile* has been used in traditional medicine for gastrointestinal remedies such as the treatment of diarrhea and colic as well as used by women for postpartum care; uterine involution, treatment of uterine pain and uterine inflammation.

Zingiber montanum is used traditionally as medicine to heal stomach discomfort, tumours, relieving rheumatic pains, and also as spice and food flavouring (Bua-in & Paisooksantivatana, 2009; Ibrahim *et al*., 2008). Young inflorescences of *Etlingera elatior* are commonly used as the ingredients of spicy dishes; post-partum women use the leaves together with other aromatic herbs for bathing to remove body odour and also used for cleaning wounds (Ibrahim *et al*., 2007; Chan *et al*., 2009). The leaves and rhizomes of *Kaempferia galanga* are used in traditional medicine, perfumery, and food flavouring. The rhizomes are used as expectorants and carminatives as well as used as ingredients for preparing 'Jamu' (Ibrahim *et al*., 2007; Chan *et al*., 2009).

2.4 MOSQUITO

Throughout human history, mosquitoes are the famous large group of insects causing great suffering on account of their blood-sucking habits and their ability to support and transmit disease-causing organisms. Almost three quarters of all mosquito species were found living in the humid tropics and subtropics (Miyagi and Toma, 2000).

They are small, 5 to 15 mm in length, long–legged, two-winged insects. The adults differ from other flies in having the following two characters in combination: an elongated mouth or proboscis and scales on the wing veins and wing margins. Both male and female mosquitoes feed on plant fluids and nectar. However, the female character requires a blood meal from a warm-blooded animal before a viable batch of eggs can be laid, and only extend about a quarter of the length of the proboscis. The female has long, needle-like mouthparts which are capable of piercing animal tissue. Male mosquitoes have a pair of long bushy (plumose) antennae, whereas the antennae of the female are sparsely haired

or pilose (Figure 2.6). Both sexes have one pair of compound eyes (Burgess & Cowan, 1993).

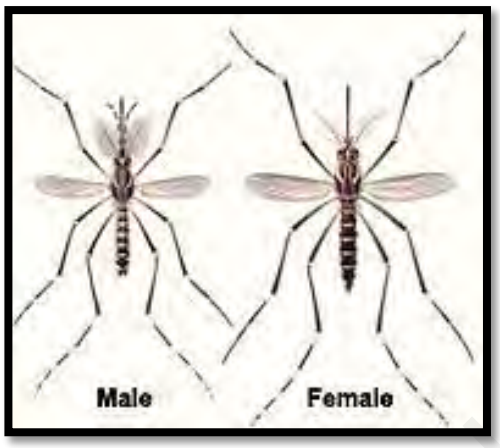


Figure 2.6: The differences between male and female mosquito (Clements, 1992)

2.4.1 *Aedes* mosquito

Aedes mosquitoes belong to the family Culicidae, suborder Nematocera of the order Diptera. *Aedes* have captured much attention in the laboratory and the field because of its importance as vector of human diseases, such as vector for dengue fever, dengue haemorrhagic fever, and chikungunya in many countries, especially in Southeast Asia countries (Chen *et al*., 2005; Noridah *et al*., 2007; Leroy *et al*., 2009).

Ae. albopictus Skuse is believed to have originated from Southeast Asia and has spread throughout Africa, Europe and America during early 20th century (Smith, 1956). This mosquito species is relatively small, black mosquito with white snowy marking on its body. It is differentiated from other *Aedes* species by its silvery line which runs down the center of the thorax.

Meanwhile, *Ae*. *aegypti* Linnaeus is presumed to be indigenous to Africa (Mattingly, 1957). This mosquito species was introduced to Southeast Asia in 1850 and in 1913, it started to become the dominant mosquito, spread in Kuala Lumpur and throughout the

country (Macdonald, 1957). *Ae*. *aegypti* is a dark brown mosquito with lyre-shaped marking on its mesonotum which is covered with silvery white scales.

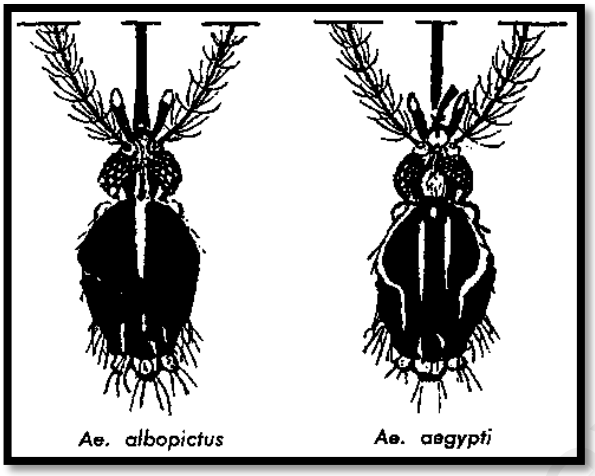


Figure 2.7: The differences between *Ae*. *albopictus* and *Ae*. *aegypti* mosquito (Clements, 1992)

2.4.2 *Culex* mosquito

Culex mosquitoes belong to the family Culicidae, suborder Nematocera of the order Diptera. Most of the *Culex* mosquitoes are nocturnal. *Cx quinquefasciatus* Say is an important urban nuisance mosquito in many parts of the world (Lee *et al*., 1997). They transmit a multitude of pathogens and become vector of urban bancroftian filariasis in Malaysia (Hardstone *et al*., 2007; Nazni *et al*., 2005; Hamdan *et al*., 2008). *Cx*. *quinquefasciatus* is brown with cross veins on narrow wings and narrow cross bands on the abdomen, which is blunt at tip. They are widely distributed in the tropical and subtropical regions in Asia, South America, the Pacific Islands and Africa.

Figure 2.8: *Culex quinquefasciatus* mosquito

2.5 LIFE CYCLE OF A MOSQUITO

Mosquitoes have four distinct stages in their life cycle. They undergo a complete metamorphosis passing through the stages of egg, larva, pupa and adult (Figure 2.9).

2.5.1 Egg

The eggs are white when first deposited before darkening to black or dark brown within an hour or two. In general there are three distinct types of mosquito eggs: (1) those that are laid singly on the surface of the water such as Anopheline eggs; (2) those that are glued together to form rafts that float on the surface of water such as in the genera *Culex*, *Culiseta*, *Mansonia*, *Uranotaenia*; and (3) those that are laid singly out of water such as the eggs of *Orthopodomyia* and some species of *Aedes* (WHO, 1972). The duration of the egg stage depends on the mosquito species and environmental conditions. The egg stage could last for one day to nine months. In the tropics including Malaysia, the mosquito eggs usually hatch within two or three days. They feed on microorganisms in the water or on the water surface using paired mouth brushes on the head.

2.5.2 Larva

The larvae of all mosquitoes live in water. Some larvae develop in permanent ponds and marshes, some in temporary flood waters or woodland pools, some in water contained in tree holes or the axils of leaves, and others in any type of artificial container that holds water. The larval stage is divided into four developmental periods known as instars. Each succeeding instar is larger than the previous. At the end of every instar, the larval shed its exoskeleton through moulting process. The larval period depends on the mosquito species and environmental factors particularly the water temperature and other variables. In warm climates like Malaysia, the larval period last about four to seven days, or longer if there is a shortage of food.

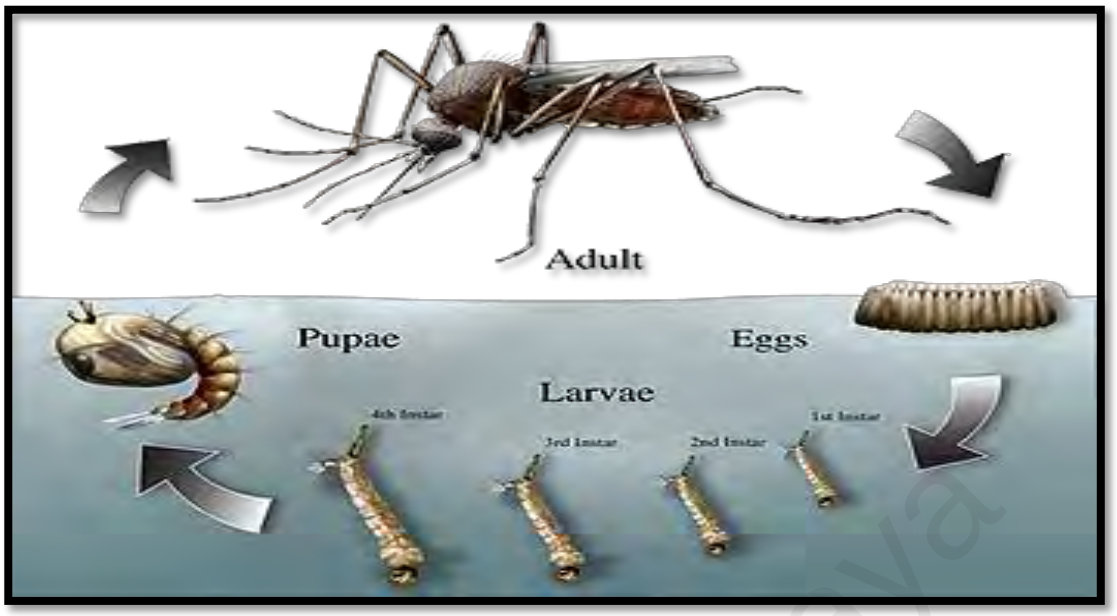


Figure 2.9: The life-cycle of mosquito (Christophers, 1960)

The larvae come to the surface to breathe using its air tube called siphon. The siphon is located at the base of the larval abdomen. The siphon uses for breathing and hang from the water surface. The larvae feed on microorganisms and organic matter in the water (Burgess & Cowan, 1993; WHO, 1972).

2.5.3 Pupa

The mosquito pupa is also aquatic and very active. Its shape and appearance are different compared to the larval stage. The body of a mosquito pupa is comma-shaped which is divided into two distinct regions: the cephalothorax and the abdomen. The cephalothorax has a pair of respiratory trumpets which is used by the pupa for breathing while the abdomen has a pair of paddle-like appendages at the tip which is used for mobile. During the pupal stage, all the larval tissues metamorphose into adult tissues but they do not need any feeding. The transformation of pupal into adult mosquito could be seen inside the pupal case as the pupa is transparent. The pupal stage lasts for a period that varies from one to three days and at the end of the pupal stage, the pupal case is broken and the adult mosquito emerges directly (Burgess & Cowan, 1993; WHO, 1972).

2.5.4 Adult

The adult mosquito is a fragile insect with a slender abdomen, a pair of narrow wings and three pairs of long, slender legs. The newly emerged adult rests on the surface of the water for a short time to allow itself to dry and all its parts to harden. Also, the wings have to spread out and dry properly before the adult mosquito can fly. Generally, male mosquitoes will emerge a few days earlier than female mosquitoes to give them enough time to mature before the emergence of female mosquitoes. The male mosquitoes use their feathery antennae to detect the wing-beat frequency of the newly emerged female mosquitoes which differ for every mosquito species. When the females emerge, mating will take place. Female mosquitoes mate only once and store the sperm in spermathecae. Male mosquitoes usually live for only a week (Burgess & Cowan, 1993; WHO, 1972).

Both male and female feed on nectars of fruit juices for their energies. However, only female mosquitoes feed on blood through biting their preferred hosts such as humans, warm-blooded bird, mammals or cold-blooded animals. *Aedes* mosquitoes persistently bite humans and mammals, mainly at dawn and in the early evening while *Culex* mosquitoes prefer birds than humans and attack at dawn or after dusk (Burgess & Cowan, 1993; WHO, 1972).

Blood meals are needed as a protein source for the egg development in female mosquitoes. Once the female mosquitoes have fully engorged, they fly to a shaded and quiet environment until their eggs are completely developed, within three to five days. Once the eggs are developed, the female mosquitoes which are known as gravid females begin their search for an appropriate place to lay their eggs. After completing the egg laying activities, these survived female mosquitoes begin searching for another blood meal before laying another batch of eggs. Female mosquitoes are generally capable of laying about one to three batches of eggs throughout their lifetime. Those female mosquitoes that obtain more than one blood meal are the ones that may transmit diseases

since they have in contact with several different hosts. These processes are repeated until the mosquito dies (Rozendaal, 1997).

2.6 HABITAT CHARACTERISTICS

Aedes breeding places are extremely variable. *Ae*. *albopictus* is most commonly found in suburban and rural areas where there are open spaces with considerable vegetation (Ponlawat *et al*., 2005). Since they are a successful colonizer, they can easily move to other locations and able to exploit habitats such as scarp yards, tires, and discarded containers (WHO, 1972; Rai, 1986).

Ae. *aegypti* is a domesticated mosquito species which prefers the clean water found in many types of domestic containers inside or near human dwellings (Chareonviriyaphap *et al*., 2003). This species is also known to oviposit in tree holes, rock holes and plant axils (Lenhart *et al*., 2005).

Cx. *quinquefasciatus* usually breeds in polluted water with high organic content, including sewage. It also breeds in the artificial containers and catchment basins of drainage systems and thrive abundantly in stagnant dirty water (WHO, 1972; Hamdan *et al*., 2005).

2.7 PHARMACOLOGICAL AND INSECTICIDAL ACTIVITIES OF SELECTED ZINGIBERACEAE SPECIES

Selected Zingiberaceous species contain a variety of compounds which showed insecticidal, oviposition, antifeedant, development modifying properties and repellent activity against many tested insects (Pitasawat *et al*., 2003; Choochote *et al*., 2005; Prajapati *et al*., 2005; Bandara *et al*., 2005). According to a report by Habsah *et al*. (2000), some less-polar constituents, such as curcuminoids, kava pyrones, and gingerols, isolated from Zingiberaceae species have been reported to possess antifungal, antioxidant,

insecticidal, and anti-inflammatory activities. In addition, antiulcer, antibacterial, anticonvulsion, antitumor, and antiallergen activities have also been reported by Ahmad *et al*. (2006). Kalaivani *et al*. (2012) added that the rhizomes of many species of *Zingiber* contain essential oils, curcuminoids and diarylheptanoids which have been shown to have medicinal properties, such as anti-inflammatory and anti-allergic.

Kamaraj *et al*. (2010) stated that the chloroform extracts of *Zingiber zerumbet* which were tested against trophozoites of *Giardia intestinalis*, showed anthelmintic activity against human *Ascaris lumbricoides*. Another study which was also conducted by Kamaraj *et al*. (2010), revealed that hexane extracts of *Z. zerumbet* showed the highest larvicide effects against fourth instar larvae of *Culex gelidus* and *Culex quinquefasciatus,* compared to ethyl acetate and methanol extracts of *Aristolochia indica* L., *Cassia angustifolia* Vahl., *Dyospiros melanoxylon* Roxb., *Dolichos biflorus* L., *Gymnema sylvestre* (Retz) Schult, *Justicia procumbens* L., and *Mimosa pudica* L.

Curcuminoids present in the rhizome of *Zingiber montanum* possess antioxidant, anti-bacterial, anti-inflammatory and anti-allergic activities (Bua-in & Paisooksantivatana, 2009; Kamazeri *et al*., 2012; Masuda & Jitoe, 1995; Ozaki *et al*., 1991). At the same time, insecticidal activity has been reported against bruchid (Coleoptera: Bruchidae) and against the second instar of *Ae. aegypti* larvae from dichloromethane extract of *Zingiber purpureum* (Bandara *et al*., 2005). The undiluted oil (with absolute ethanol) of *Zingiber cassumunar* exhibited 100% repellency against the larvae of *Leptotrombidium* chiggers (Acari: Trombiculidae), the vector of scrub typhus (Eamsobhana *et al*., 2009).

Essential oils derived from leaf and rhizome of *Z. officinale* var. *rubrum* showed antibacterial activity such as reported by Sivasothy *et al*. (2011). Further, Pushpanathan *et al*. (2008) studied the essential oil of *Zingiber officinale* as a larvicidal and repellent agent against the filarial vector *Culex quinquefasciatus*. Another report by Thavara *et al*.

(2007) also found that the essential oil extracted from *Z. officinale* showed repellency activity against three cockroach species: *Periplaneta americana*, *Blatella germanica*, and *Neostylopyga rhombifolia*.

Tawatsin *et al*. (2001) have studied the volatile oils extracted by steam distillation from turmeric (*Curcuma longa*), kaffir lime (*Citrus hystrix*), citronella grass (*Cymbopogon winterianus*) and hairy basil (*Ocimum americanum*). They found that those volatile oils demonstrate potential as topical repellents against both day- and night-biting mosquitoes (*Aedes aegypti*, *Anopheles dirus* and *Culex quinquefasciatus*). Moreover, Pitasawat *et al*. (2003) investigated the ethanol extracts of *Curcuma aeruginosa*, *Curcuma aromatica*, and *Curcuma xanthorrhiza* against *Aedes togoi* under laboratory conditions. The result showed that only *Curcuma aromatica* extract possessed repellency activity. A previous study by Sivasothy *et al*. (2013) reported that flavonoids and curcuminoids from *Zingiber spectabile* showed antioxidant and antibacterial activity. However, as far as our literature survey could ascertain, there is no report on insecticidal activity of *Z. spectabile*.

Many studies on the mosquitocidal activity have been reported. For examples: β-caryophyllene which was identified in *Copaifera multijuga* and *Hymenaea courbaril*; 4-gingerol, (6)-dehydrogingerdione and (6)-dihydrogingerdione which were isolated from the petroleum ether extract of the rhizome of *Zingiber officinale*; and zingiberene, citronellol and β-sesquiphellandrene which found as the major compounds in *Z. officinale* essential oil, showed effective larvicidal activity against *Ae. aegypti* (Rahuman *et al*., 2008; Aguiar *et al*., 2010; Tavares *et al*., 2013;). In addition, Lin *et al*. (2010) found that the pure secondary metabolites from the roots of *Z. officinale* including [10]-shogaol, [6]-gingerol, [10]-gingerol and [6]-shogaol, have larvicidal activity against the parasitic round worm, *Angiostrongylus cantonensis*.

CHAPTER 3: MATERIALS AND METHODS

3.1 PLANT MATERIALS

Rhizomes of *Zingiber officinale* var. *rubrum*, *Zingiber montanum*, *Zingiber zerumbet* and *Curcuma aeruginosa* (20 kg each) were purchased from the local market while rhizomes of *Zingiber spectabile* (20 kg) were collected from University Malaya Field Station, Gombak and all species were authenticated by Prof. Halijah Ibrahim.

3.2 PREPARATION OF SAMPLE

3.2.1 Preparation of the rhizome powder

The rhizomes of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* were washed, dried at room temperature for one day then cut into pieces (Figure 3.1), finely ground and dried at 60°C (Figure 3.2) for three days to get dried fine powder (Figure 3.3).

3.2.2 Preparation of plant extracts

In this study two different extracts were prepared: hexane extract and dichloromethane extract. To obtain hexane extract, 500 g of the rhizome powder of each ginger species were soaked in ± 2.5L hexane solvent (99% AR Systerm) in a conical flask separately (Figure. 3.4). The extraction was left for 3 days. Later, the extract was filtered (Figure 3.5) and was allowed to evaporate using rotary vacuum evaporator (Buchi Rotavapor R-114, Switzerland) until all solvent evaporated (Figure 3.6).

Then the extract was transferred into 7 ml vials, labelled, and the weight was recorded (Figure 3.7). The vials were then covered with aluminum foil and kept in -4°C until further tests.

Figure 3.1: The rhizomes of *Z. officinale* var. *rubrum* were sliced into pieces

Figure 3.2: The rhizomes were dried at 60^{o}C

Figure 3.3: The rhizome powder of *Z. officinale* var. *rubrum*

Figure 3.4: The rhizome were soaked with hexane solvent

Figure 3.5: The filtration of the extract

Figure 3.6: The extract were evaporated using rotary vacuum evaporator

Figure 3.7: The *Z. zerumbet* extract was transferred into the vial

In order to obtain dichloromethane extract, the rhizomes of each plant species were soaked in dichloromethane solvent (99% AR Systerm) and similar extraction process were repeated.

The percentage of extraction was calculated by using the following formula:

$$\% \text{ extraction} = \frac{\text{Weight of the extract}}{\text{Weight of the plant material}} \text{ x } 100\%$$

Further, the hexane and dichloromethane extracts were screened for the presence of chemical constituents.

3.3 GAS CHROMATOGRAPHY – MASS SPECTROMETRY

Samples of plant extracts were diluted in hexane and dichloromethane separately (6:100) and analyzed on an Agilent GC-MSD apparatus equipped with 5973 N mass selective detector and HP-5(5% phenyl methylpolysiloxane) capillary column. The oven temperature was programmed from 50^0C to 280^0C at the rate of 4 C/min and held at this temperature for 5 min. The injector and interface temperatures were 250^0C and 280^0C, respectively. The carrier gas was helium at a flow rate of 1.0 ml/min (constant flow). The sample (20 µl) was injected with a split of 20:1. Electron impact mass spectrometry was carried out at 70 eV. The ion source and quadrupole temperatures were maintained at 230^0C and 150^0C respectively.

Figure 3.8: The gas-chromatography and mass spectrometry machine

3.4 MOSQUITO CULTURE

3.4.1 The larvae

The larvae of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* were obtained from the Insectary of Medical Entomology Unit, Institute for Medical Research (IMR), Kuala Lumpur. The eggs were immersed in dechlorinated tap water for hatching. The first and second instar larvae of *Ae. albopictus* and *Ae. aegypti* were fed with liver powder while the third and fourth instar were fed with small chunks of half cooked liver. However, the first to fourth instar larvae of *Cx. quinquefasciatus* were fed with mice chew. In this study, the late third instar and early fourth instar larvae were used for the larvicidal bioassay experiment.

Figure 3.9: Third-instar of *Cx. quinquefasciatus* larvae

3.4.2 The adult

The adult mosquito of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* were also obtained from the Insectary of Medical Entomology Unit, Institute for Medical Reseacrh (IMR), Kuala Lumpur. They were provided with 10% sucrose solution fortified with 1% vitamin B complex. The colony was maintained in the insectarium at 25±27^{o}C and 62±80% RH with 12:12 h (L:D) photoperiod regime. The female adults aged 4-7 days were used for adulticidal bioassay and the tunnel experiments.

Figure 3.10: The adult mosquitoes were provided with 10% sucrose solution

3.5 LARVICIDAL BIOASSAY

Larvicidal bioassays were performed by following the WHO guidelines (2005) with slight modification. Each of the extracts (0.6 mg) was dissolved in 100 ml methanol as stock solution. Various concentrations were prepared (70 mg/L, 100 mg/L, 200 mg/L, and 300 mg/L) from the stock solution and dispensed separately into testing cups containing 200 ml of dechlorinated water. Then, a total of 25 late third instar larvae were introduced into each testing cup and the water was top up until 250 ml. Each concentration were prepared in triplicate. Methanol served as negative control and temephos as positive control. The experiment were carried out at room temperature (25±27ºC; 62±80% RH).

Figure 3.11: Example of larvicidal bioassays of hexane extract of *Z. zerumbet* against larvae of *Cx. quinquefasciatus*

Knockdown (KD) and mortality of the larvae were also observed. Knockdown is defined as the initial effect such as morbid or unusual behaviour due to the alteration of a specific physiological process that has taken place upon contact with the toxicant (Hidayatulfathi *et al*., 2004). According to WHO (2016), KD and mortality is different in terms of the time of observation. The assessment of KD was less than 24 hours after exposure (1h, 3h and 6h) while mortality was determined at least after 24 hours after exposure. Larvae were considered as KD when the larvae showed sign of unnatural restlessness, wriggling movement, frequent sinking followed by floating, sluggishness with random tremor at the bottom of the container and paralyzed (Ahmad *et al*., 2016). Larvae were considered as dead when they did not respond to the stimuli such as probing with a needle in the siphon or cervical region, incapable of rising to the surface of the water (within a reasonable period of time) or showing a characteristic diving reaction when the water disturbed, display discolouration, unnatural positions, and incoordination or rigor (Komalamisra *et al*., 2005). Experiments were done in three replicates and the percentage mortality was reported from the average of three replicates.

3.6 ADULTICIDAL BIOASSAY

3.6.1 Preparation of solution and impregnated paper

Each of the extract (1.8 mg) was dissolved in 3 ml methanol to make a concentration of 1000 mg/m^2. Then it was applied on filter paper (size 12 x 15 cm) bought from Vector Control Research Unit, University of Science Malaysia (USM). Control papers were treated with methanol under similar conditions. Then the paper was left to dry at room temperature (25±27^{o}C; 62±80% RH) for about one hour (Figure 3.12). Then it was covered with aluminum foil and kept in refrigerator (-4^{o}C) until further use (Figure 3.13, left).

3.6.2 The adulticidal bioassay

The adulticidal bioassays followed WHO (1981) protocol. This method used a special tube namely the holding chamber and the exposure chamber. In the holding chamber, a piece of clean white paper was rolled into a cylinder and fastened with a silver clip to mark the wall. Then the impregnated paper was rolled and clipped at the exposure chamber (Figure 3.13, right). A total of fifteen female mosquitoes (5-6 days post emergence, blood fed) was used in this study.

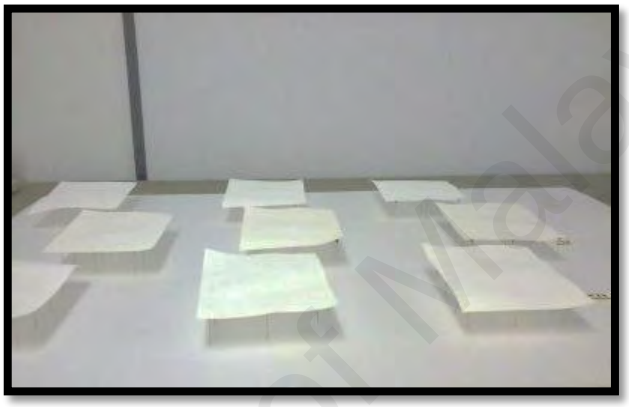


Figure 3.12: The impregnated papers were left to dry at room temperature

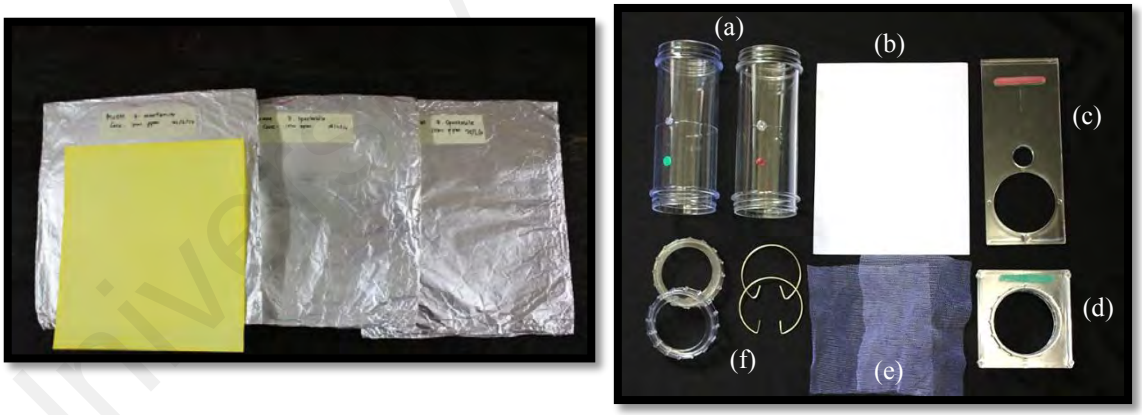


Figure 3.13: The examples of impregnated paper of the dichloromethane extracts of *Z. montanum* (left); Equipment used for the adulticidal bioassays: (a) the green tube/holding chamber and red tube/exposure chamber, (b) impregnated paper, (c and d) sliding door, (e) net, (f) clip (right)

After that, the mosquito were introduced to the holding chamber and let it acclimatized. After 30 minutes, the doors of both chambers were opened. The exposure tube was left standing upright and the partition removed to allow free flow (Figure 3.14). Then the tubes were covered with black cloth during the experiment (Figure 3.15). The

mosquitoes were exposed to the test paper for 3 h and every 15 minutes the knockdown was recorded throughout the experiment.

At the end of the exposure period, the mosquitoes were transferred back to the holding tube and kept for 24 h for the recovery period. A pad of cotton soaked in 10% glucose solution was placed on the mesh screen (Figure 3.16). Each experiment was replicated three times together with appropriate control. Mortality of mosquitoes was determined at the end of 24 h recovery period. The temperature was maintained throughout the experiment at 25±27ºC; 62±80% RH.

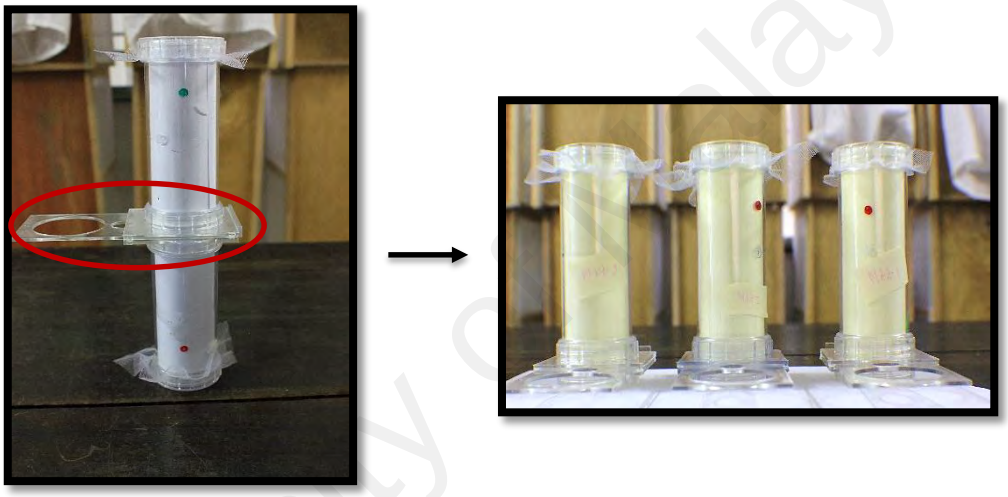


Figure 3.14: The door was opened after the mosquito acclimatized for 30 minutes and the holding chamber was removed

Figure 3.15: The exposure tube were covered with the black cloth for 3 h exposure

Figure 3.16: A pad of cotton soaked in 10% glucose solution was placed on the mesh screen after the exposure period

3.7 TUNNEL TEST EXPERIMENT

3.7.1 Preparation of solution and impregnated net

A total of 4.8 mg crude extract was dissolved in 5 ml methanol to make a concentration of 1000 mg/m^2. Then it was applied to the net (size 20 x 24 cm). Control nets were treated with methanol under similar conditions. Both impregnated nets and control nets were left to dry at room temperature (25±27^{o}C; 62±80% RH) (Figure 3.17). Then the impregnated nets were covered with aluminum foil and kept in refrigerator (-4^{o}C) until further use (Figure 3.18)

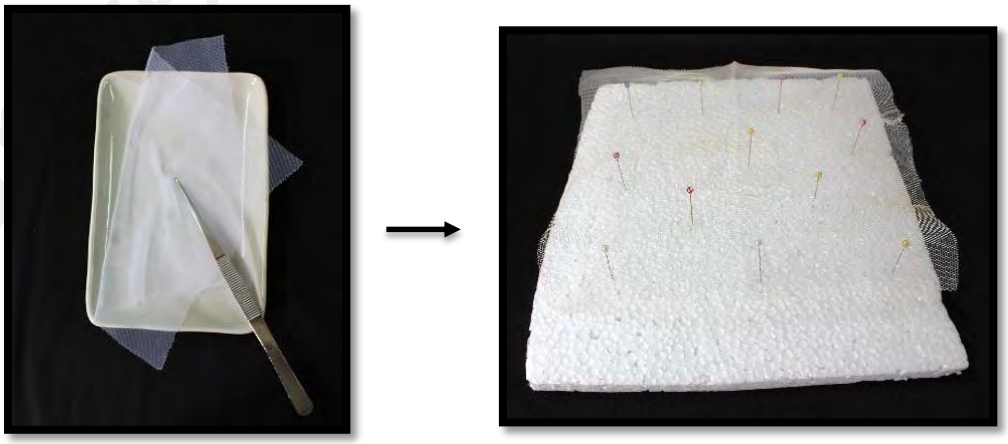


Figure 3.17: The net was dissolved in extract (left) and dried at room temperature (right)

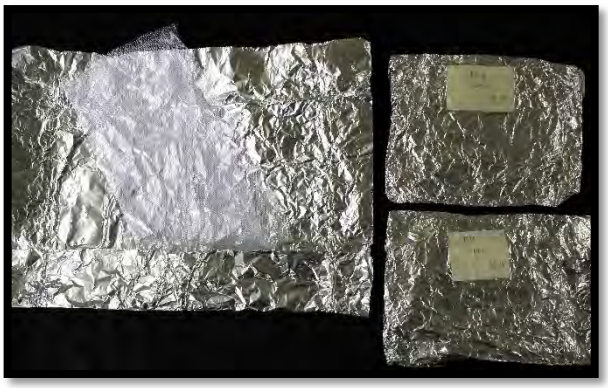


Figure 3.18: The nets were covered with aluminum foil

3.7.2 Tunnel test procedures

The tunnel test is used to measure the mortality and blood-feeding success of the host-seeking mosquitoes in an experimental chamber. The assay is carried out in a laboratory by releasing non-blood-fed female mosquitoes aged 5-8 days into a 75cm tunnel made of glass (Figure 3.19). At each end of the tunnel, a 28cm^2 cage covered with polyester netting was fitted (extension). The surface of the netting available to the mosquitoes is 480 cm^2 (20 cm x 24 cm), with nine holes 1 cm in diameter (Figure 3.20).

In the shorter section of the tunnel, a mouse was placed, which was unable to move and was available for mosquito biting (Figure 3.21). A total of twenty-five female mosquitoes was introduced into the cage at the end of the longer section of the tunnel. A tunnel with untreated netting is always used as a control. During the tests, all the tunnels were covered with black cloth maintained at 25±27^{o}C; 62±80% RH. Each test was replicated three times together with control and the mortality was recorded after 24 h (Figure 3.22).

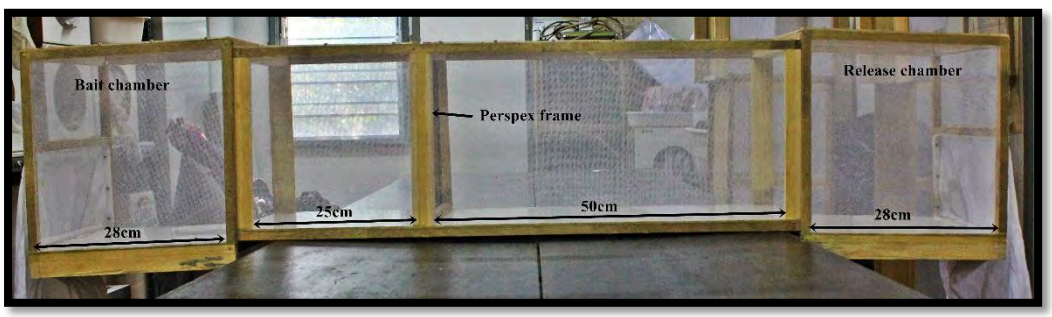


Figure 3.19: Tunnel for the test

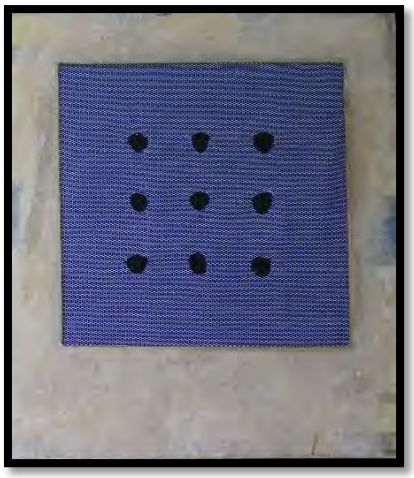


Figure 3.20: The net attached in the perspex frame

Figure 3.21: The mouse

Figure 3.22: The tunnels were covered with black cloth during the test

3.8 STATISTICAL ANALYSIS

3.8.1 Larvicidal activity

The average larval mortality of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* against the hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* were recorded using excel spreadsheet. In cases where the control mortality was between 5%-20%, the observed percentage mortality (%M) was corrected by Abbott's formula (Abbott 1925):

$$\%M = \frac{\%\text{ test mortality} - \%\text{ control mortality}}{100 - \%\text{ control mortality}} \times 100\%$$

Due to the "not normal distribution of this variable", Generalized Linear Model (GLZ) was applied with three source of variations and interactions: *Zingiber* species, concentrations and exposure time. Least Significant Difference (LSD) Fisher test was used for post hoc analyses. Results with $p<0.05$ were considered to be significantly different. The LC_{50} & LC_{90} values (with 95% confidence limits), chi-square values, regression coefficient, slope and standard error were calculated by probit analysis using Statistical Package for Social Science (Version 21).

The Zingiberaceae species extracts in this present study will be categorized according to Komalamisra *et al*. (2005) where the extracts with LC_{50} values < 750 mg/L were considered effective, extracts with 50 mg/L $<LC_{50}<$ 100 mg/L were considered moderately effective and those with LC_{50} values < 50mg/L were considered highly effective.

3.8.2 Adulticidal activity

The percentage mortality of adult *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* when exposed to the hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z.*

montanum, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* were calculated using excel spreadsheet.

Nonparametric tests: two or more related samples (Friedman's 2-way ANOVA by ranks) was used to identify if there are any significant differences between percentage mortality of the mosquito species and the exposure time. Results were considered to be significantly different when $p<0.05$. Besides that, the regression equation and regression values were presented to predict the numbers of mosquito which died and to show the relationship between exposure time and percentage mortality.

3.8.3 Repellency activity

In the tunnel test, the percentage of blood feeding inhibition, repellence and mortality were also computed using excel spreadsheet. Since the data was not normally distributed, the nonparametric tests: Two or more independent samples (Kruskal-Wallis 1-way ANOVA) was used to identify whether there is any significant difference on repellency percentage, blood feeding percentage and mortality percentage of the three mosquito species with the plant extracts and exposure time. The results were significantly different when $p<0.05$. The lethal time (LT_{50} and LT_{90}) were also calculated using probit analysis using Statistical Package for Social Science (Version 21).

CHAPTER 4: RESULTS

4.1 YIELD OF THE PLANT EXTRACTS

The percentage yield of five Zingiberaceae species extracted using two organic solvents: hexane and dichloromethane are presented in Table 4.1.

Table 4.1: Yield of the hexane and dichloromethane extracts from five Zingiberaceae species

Plant species	Weight of crude extract (%)	
	Hexane	Dichloromethane
Zingiber officinale var. *rubrum*	1.72	1.57
Zingiber montanum	2.47	4.50
Zingiber spectabile	1.72	1.37
Zingiber zerumbet	4.33	5.11
Curcuma aeruginosa	3.19	5.90

Plant samples were extracted using hexane and dichloromethane. The hexane extract of *Z. zerumbet* showed the highest yield percentage of 4.33%, followed by the hexane extract of *C. aeruginosa* (3.19%) and the hexane extract of *Z. montanum* (2.47%). The hexane extract of *Z. officinale* var. *rubrum* and *Z. spectabile* gave the same yield percentage at 1.72%.

On the other hand, *C. aeruginosa* extracted using dichloromethane yielded 5.90% which is the highest extract yield compared to the other species. *Z. zerumbet* gave 5.11%, followed by *Z. montanum* (4.50%), *Z. officinale* var. *rubrum* (1.57%) and *Z. spectabile* (1.37%).

Based on the yield recovered from the hexane and dichloromethane plant extracts, *Z. zerumbet* gave the highest yield of extract from both solvents (9.44%). This was followed by *C. aeruginosa* (9.09%) and *Z. montanum* (6.97%). Extracts from *Z. officinale* var. *rubrum* showed low yield at 3.29% and *Z. spectabile* (3.09%) was the lowest.

The hexane and dichloromethane extracts of five Zingiberaceae species used in the study are represented by acronyms as in Table 4.2.

Table 4.2: Acronym of Zingiberaceae species used in the study

Plant Species	Hexane extract	Dichloromethane extract
Z. officinale* var. *rubrum	HZOR	DZOR
Z. montanum	HZM	DZM
Z. spectabile	HZS	DZS
Z. zerumbet	HZZ	DZZ
C. aeruginosa	HCA	DCA

4.2 CHEMICAL ANALYSIS OF THE FIVE ZINGIBERACEAE SPECIES

All extracts obtained from the plants were analyzed using Gas Chromatography - Mass Spectrometry (GC-MS). The chemical analysis on the hexane extracts obtained from rhizomes of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* led to the identification of 43, 82, 50, 51, and 74 compounds, respectively. The 5 major compounds identified in each of Zingiberaceae species are listed as in Table 4.3.

The highest percentage of compounds obtained from each of the hexane extracts were zingerone with 14.92% in HZOR; benzoic acid (17.20%) in HZM and 1,1'-Ethylenebisdecalin (40.21%) in HZS. Zerumbone was found to be the most abundant compound recorded in HZZ with 60.40% while trans-dihydrocarvone (13.92%) was recorded as the major compound in HCA. In addition, zerumbone was not only identified in HZZ (60.40%) but also found in HCA in trace amount (2.98%).

Table 4.3: The major compounds of the hexane extracts of five Zingiberaceae species

Plant Species	Total Compounds	Major Compounds	% of compounds
Z. officinale* var. *rubrum	43	Zingerone	14.92
		Benzaldehyde dimethyl thiol acetal	11.61
		Zingiberene	8.62
		Gingerol	7.20
		β-sesquiphellandrene	5.81
Z. montanum	82	Benzoic acid	17.20
		6,7-Dimethoxyquinoxaline	15.49
		Ethanone,1-[5-(2-furanylmethyl)-2-furanyl]-	14.20
		2,4,5-trichlorophenol	10.32
		Terpinen-4-ol	2.86
Z. spectabile	50	1,1'-Ethylenebisdecalin	40.21
		1-Pentadecyne	11.50
		2-oleoylglycerol	1.57
		Allomadendrene oxide-(I)	1.37
		Allopregnanolone	1.28
Z. zerumbet	51	Zerumbone	60.40
		Humulene epoxide II	20.84
		1,1'-Ethylenebisdecalin	8.33
		α-caryophyllene	6.90
		Camphene	1.38
C. aeruginosa	74	Trans-dihydrocarvone	13.92
		Tropolone	11.93
		Zerumbone	2.98
		Amiphenazole	2.45
		Camphor	1.29

Table 4.4: The major compounds of the dichloromethane extracts of five Zingiberaceae species

Plant Species	Total Compounds	Major Compounds	% of compounds
Z. officinale* var. *rubrum	43	Zingerone	19.94
		9-O-methylnorbelladine	16.47
		Capsaicin	5.51
		Zingiberene	4.70
		α-curcumene	2.87
Z. montanum	70	Carbendazim	26.58
		2,4,5-trichlorophenol	19.59
		Dimethyl 4-methylphthalate	12.64
		Trans-2,5-dimethoxy cinnamic acid	12.64
		Terpinen-4-ol	2.56
Z. spectabile	60	1,1'-Ethylenebisdecalin	22.60
		Dihydrochondrillasterol	12.92
		2-monopalmitin	3.11
		2-linoneoyl glycerol	2.82
		2-monoolein	2.68
Z. zerumbet	44	Zerumbone	47.77
		α-caryophyllene	7.05
		3-bromo-1-cyclodecene	6.63
		Humulene oxide	2.88
		Camphene	2.74
C. aeruginosa	58	2-Pyridinamine,4,6-dimethyl-	12.63
		Trans-dihydrocarvone	10.35
		Urea,N,N'-bis (trimrthylsilyl)	8.68
		Amiphenazole	2.20
		Eucalyptol	1.89

The GC-MS analysis on the dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* revealed 43, 70, 60, 44 and 58 compounds, respectively (Table 4.4). The results show that the major compound found in

DZOR was zingerone (19.94%), carbendazim (26.58%) in DZM and 1,1'-Ethylenebisdecalin (22.60%) in DZS. Zerumbone (47.77%) and 2-Pyridinamine,4,6-dimethyl- (12.63%) were the main compounds obtained in DZZ and DCA, respectively.

There are several common compounds that are present in both hexane and dichloromethane extracts of five Zingiberaceae species used in this study. For example: zingerone and zingiberene were identified in both HZOR and DZOR. DZOR (19.94%) exhibited higher amount of zingerone than HZOR (14.92%) while higher amount of zingiberene was reported in HZOR (8.62%) than in DZOR (4.70%). In HZM and DZM, the common components were 2,4,5-trichlorophenol which was less abundant in HZM (10.32%) compared to DZM (19.59%) and terpinen-4-ol which slightly more abundant in HZM (2.86%) than in DZM (2.56%). 1,1'-Ethylenebisdecalin was the most common constituent in both HZS and DZS with higher quantity recorded in HZS (40.21%) than in DZS (22.60%). The common compounds identified from HZZ and DZZ were zerumbone and α-caryophyllene. Higher quantity of zerumbone was recorded in HZZ (60.40%) than in DZZ (47.77%) whereas α-caryophyllene was recorded slightly abundant in DZZ (7.05%) than in HZZ (6.90%). Both HCA and DCA showed the common components of trans-dihydrocarvone and amiphenazole. The amount of the two components were higher in HCA (13.92% and 10.35%, respectively) than in DCA (2.45% and 2.20%, respectively).

4.3 LARVICIDAL BIOASSAYS

4.3.1 Evaluation of the hexane extracts of five Zingiberaceae species against *Ae. albopictus* larvae

The larvicidal activity of the hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA) against *Ae. albopictus* larvae at various concentrations and different exposure time are shown in Figure 4.1. After 1 h exposure, only HZM exhibited KD (knockdown) activity of *Ae. albopictus* larvae at all the concentrations but with low KD activity (< 3.00%) whereas hexane extracts of the other four species showed KD activity of the larvae at specific concentrations. For instance, HZOR exhibited KD activity of the larvae at 200 mg/L and 300 mg/L (4.00% and 13.33%); HZS and HZZ at 300 mg/L (1.33%) whereas there is no KD activity noted for HCA after 1 h exposure.

At 3 h exposure, HZOR showed high KD activity of *Ae. albopictus* larvae at 300 mg/L (85.33%) whereas moderate KD activity of the larvae was recorded in HZOR at 200 mg/L (46.68%) and in HZM at 200 mg/L and 300 mg/L (28.00% and 53.33%). Low KD activity of *Ae. albopictus* larvae was reported in HZOR at 70 mg/L (10.68%) and 100 mg/L (8.00%); in HZS at 100 mg/L (1.33%) and 300 mg/L (8.00%); in HZZ at 200 mg/L (4.00%) and 300 mg/L (16.00%); and in HCA at 300 mg/L (12.00%).

After 6 h exposure, only HZOR showed 100.00% KD activity of *Ae. albopictus* larvae at 300 mg/L while HZM (at 300 mg/L) and HZOR (at 200 mg/L) showed relatively high KD activity of the larvae (89.33% and 80.00%) . Moderate KD activity of the larvae was recorded in HZZ at 300 mg/L (69.33%), in HZOR at 200 mg/L (60.00%) and in HZS at 300 mg/L (48.00%). Relatively low KD activity of the larvae was reported in HZZ at 300 mg/L (30.68%), in HZM at 200 mg/L (28.00%), in HCA at 300 mg/L (22.67%) and in HZOR at 70 mg/L and 100 mg/L (21.33% and 20.00%). The lowest KD activity was

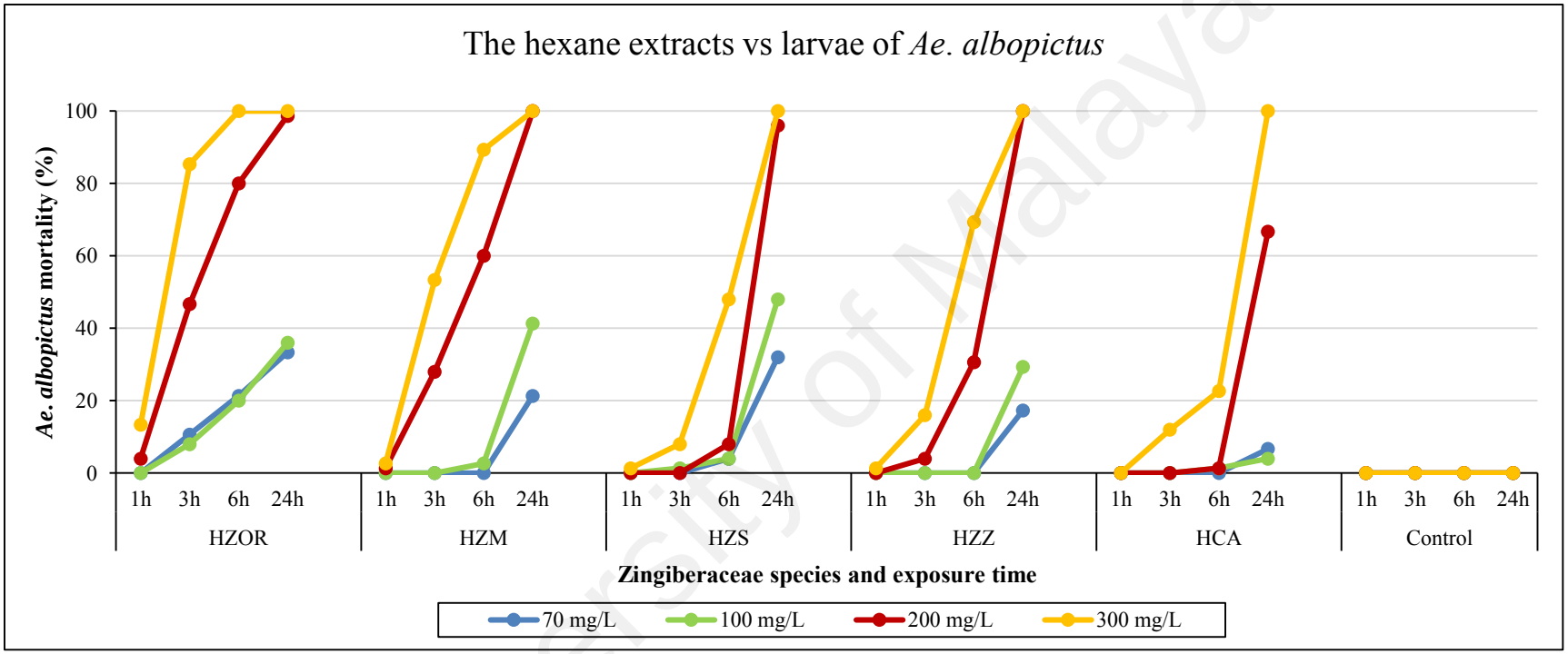
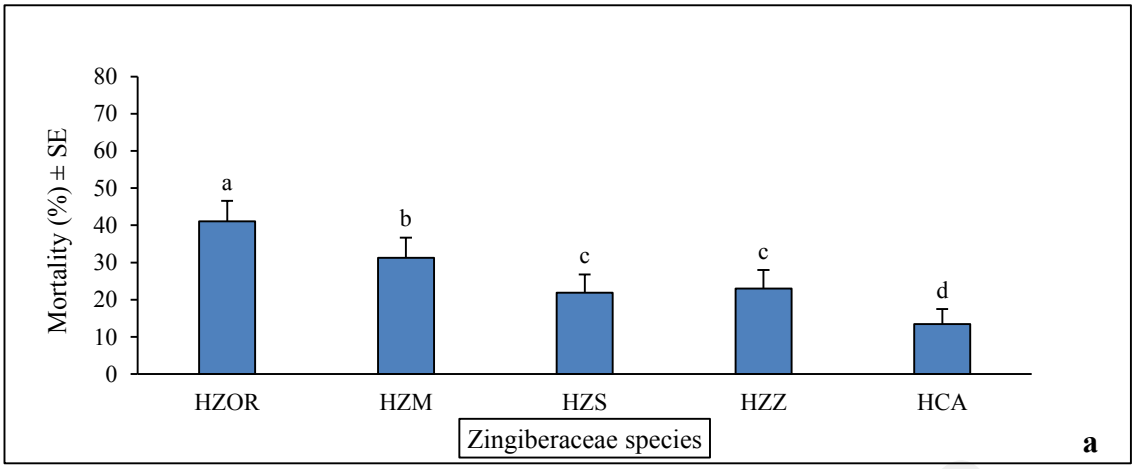


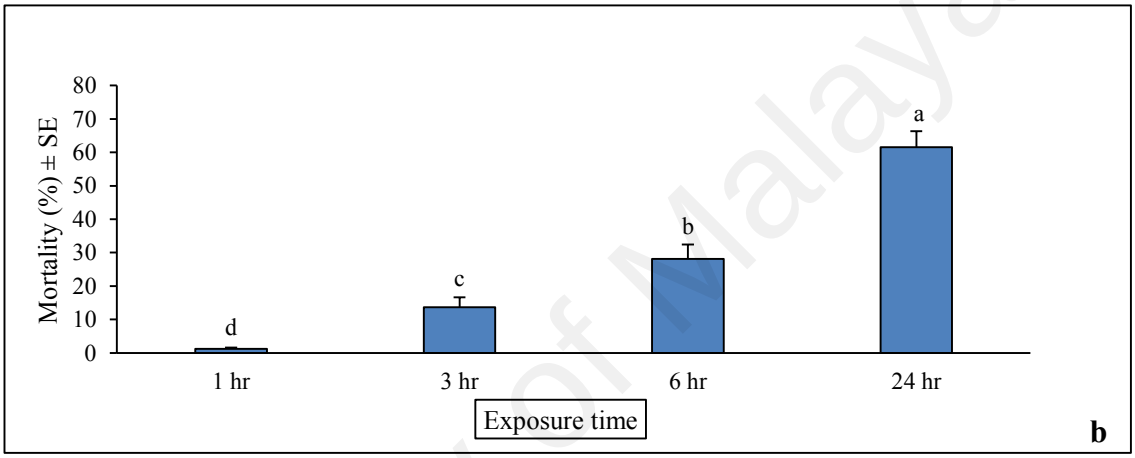
Figure 4.1: The larvicidal activity of hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA) against larvae of *Ae. albopictus* at different exposure time with various concentrations

observed in HZM at 100 mg/L (2.68%), in HZS at 70 mg/L (4.00%), 100 mg/L (4.00%) and 200 mg/L (8.00%), and in HCA at 100 mg/L and 200 mg/L (1.33%).

After 24 h observation, 96.00% to 100.00% mortality of *Ae*. *albopictus* larvae was reported at 200 mg/L and 300 mg/L in all the hexane extracts of five Zingiberaceae species, except in HCA which only showed 66.67% at 200 mg/L. Moderate percentage mortality of *Ae*. *albopictus* larvae was observed at 70 mg/L in HZOR (36.00%) and HZS (48.00%); and also observed at 100 mg/L in HZOR, HZM and HZS with 48.00%, 41.33% and 32.00%, respectively. HZM at 70 mg/L and HZZ at 70 mg/L and 100 mg/L shown relatively low mortality of *Ae*. *albopictus* larvae (21.33%, 29.33% and 17.33%, respectively) while HCA exhibited the lowest mortality of *Ae*. *albopictus* larvae with 6.67% and 4.00% at 70 mg/L and 100 mg/L. No KD and mortality activities were observed in the control group for all the exposure time.

Generalized Linear Model results showed that there was significant effects caused by different Zingiberaceae species ($F= 989.80$, $df= 4$, $p<0.05$), different time periods ($F= 5733.27$, $df= 3$, $p<0.05$), different concentrations ($F= 3798.89$, $df= 3$, $p<0.05$) and their interactions ($F= 569.42$, $df= 36$, $p<0.05$). Figure 4.2 showed LSD Fisher post hoc test results. The highest percentage mortality of *Ae*. *albopictus* larvae was observed in HZOR, followed by HZM, then HZZ and HZS, while the lowest percentage mortality was observed in HCA. Percentage mortality of *Ae*. *albopictus* larvae was significantly different between exposure time and concentrations. Concentration of 300 mg/L showed the highest mortality of the larvae compared to the others.





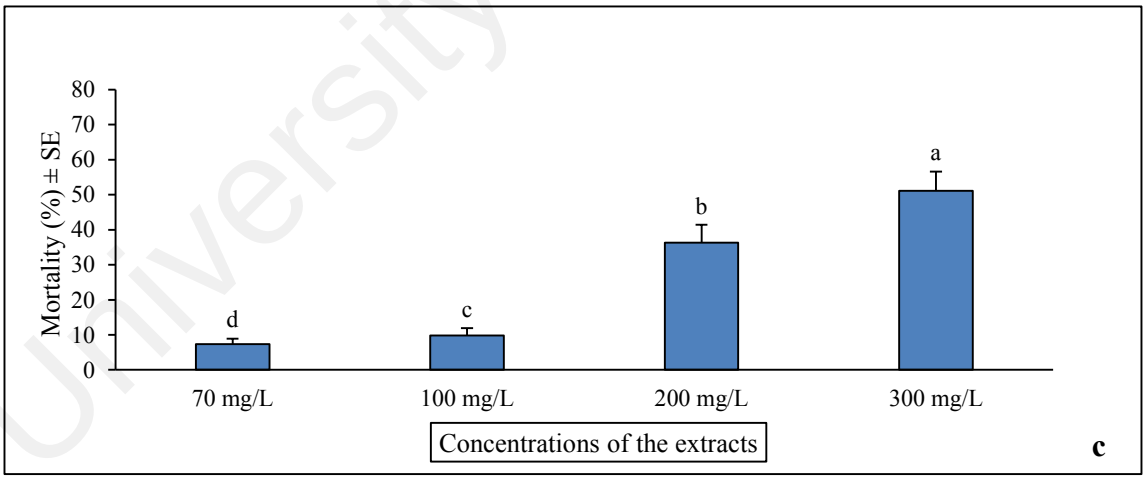


Figure 4.2: The mean percentage mortality of *Ae. albopictus* larvae after exposure to the hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA). Means with *same letters above bars* indicate no significant difference ($p<0.05$) (**a** different Zingiberaceae species, **b** different exposure time and **c** different concentrations)

Overall, the hexane extracts of five Zingiberaceae species tested in this study exhibited 100% mortality of *Ae*. *albopictus* larvae at different concentrations and exposure time. HZOR was effective in killing the *Ae*. *albopictus* larvae at 300 mg/L after 6 h exposure. HZM and HZZ induce 100% mortality of the larvae at 200 mg/L after 24 h exposure whereas HZS and HCA kill the larvae at 300 mg/L after 24 h exposure. Therefore, among these hexane extracts, HZOR was found to be the most effective in killing the *Ae*. *albopictus* larvae.

4.3.2 Evaluation of the hexane extracts of five Zingiberaceae species against *Ae. aegypti* larvae

The larvicidal activity of the hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA) against *Ae*. *aegypti* larvae were displayed in Figure 4.3. At 1 h exposure, these hexane extracts did not exhibit KD activity at all the concentration except for HZM at 100 and 300 mg/L (1.33% and 5.33%). After 3 h exposure, there was no KD activity observed in all the hexane extracts at 70 mg/L while at 100 mg/L, only HZZ exhibited KD activity of the larvae (1.33%). At 200 mg/L, HZZ showed high KD activity of *Ae*. *aegypti* larvae (88.00%) compared to HZOR (14.67%) and HZM (17.33%) whereas HZS and HCA did not show any KD activity. At the highest concentration (300 mg/L), high KD activity was reported in HZZ (96.00%); moderate KD activity was recorded in HZOR (45.33%), HZM (54.67%) and HCA (56.00%) whereas low KD activity was detected in HZS (9.33%).

After 6 h exposure, the KD activity of *Ae*. *aegypti* larvae at the concentrations of 70 mg/L to 100 mg/L ranged from 0.00% to 10.67%, respectively. At 200 mg/L, HZS showed low KD activity of the larvae (10.67%) while HZOR and HZM showed moderate KD activity (41.33% and 38.67%) and HZZ showed high KD activity of *Ae*. *aegypti* larvae (96.00%).

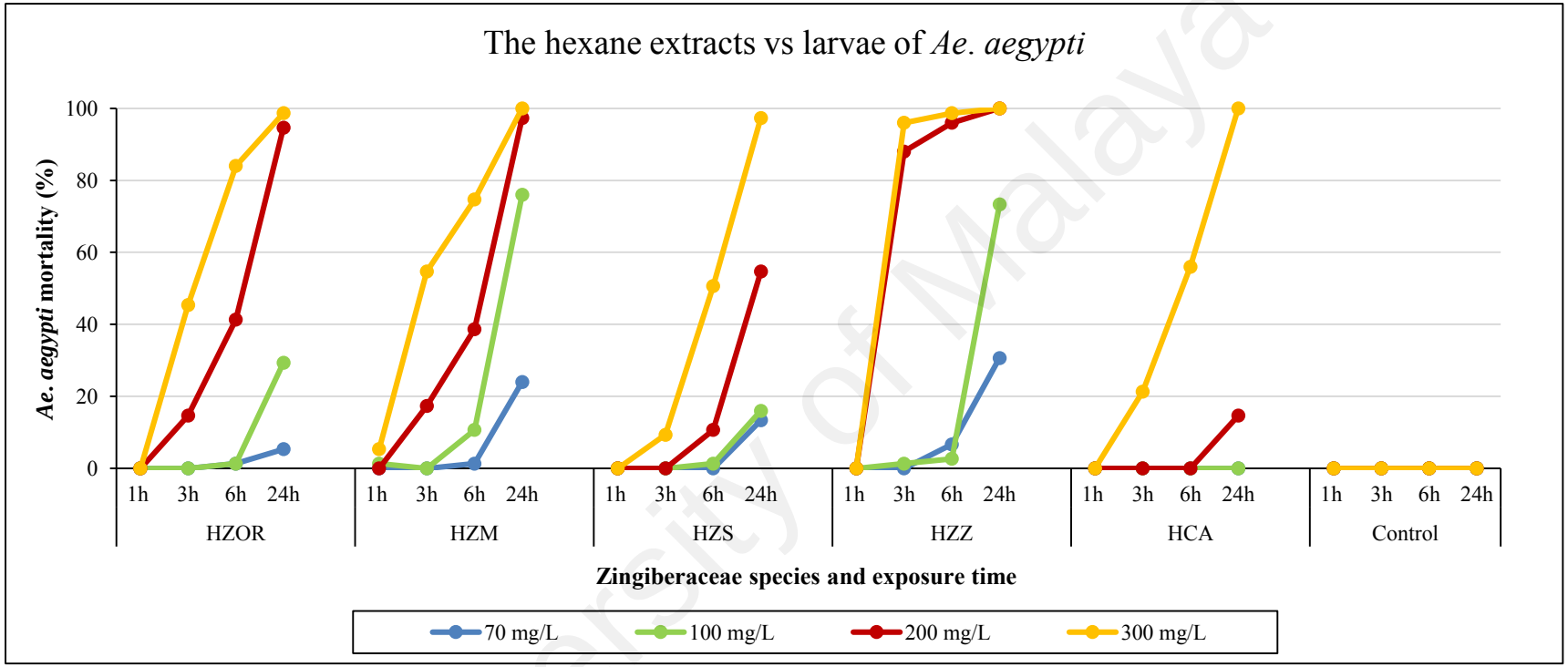


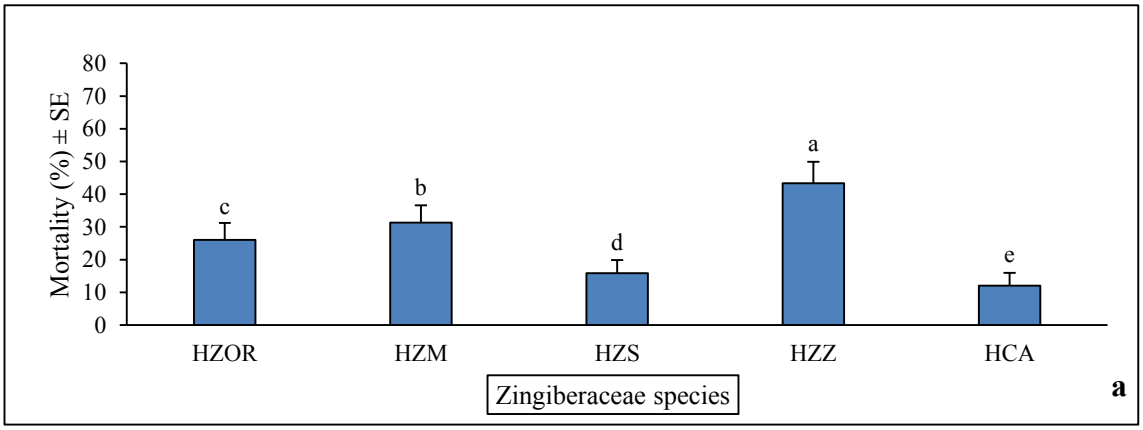
Figure 4.3: The larvicidal activity of hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA) against larvae of *Ae. aegypti* at different exposure time with various concentrations

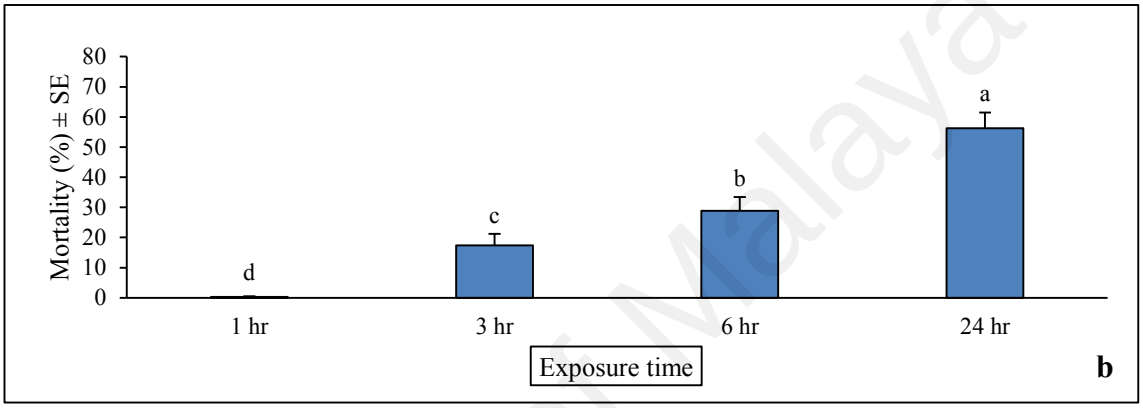
Observations at 24 h exposure showed that all of the five Zingiberaceae hexane extracts exhibited larvicidal activity at all the concentrations, except for HCA at 70 mg/L and 100 mg/L. At 70 mg/L, the KD activity of *Ae*. *aegypti* larvae in HZOR, HZM, HZS and HZZ ranged from low to much lower mortality (5.33%, 24.00%, 13.33% and 30.67%) while at 100 mg/L, the KD activity ranged from moderate to high mortality (29.33%, 76.00%, 16.00% and 73.33%, respectively). At 200 mg/L, HCA showed the lowest mortality activity with only 14.67%; HZS showed moderate activity (54.67%); HZOR, HZM and HZZ showed high mortality activity (94.67% - 100.00%). At 300 mg/L, all *Ae*. *aegypti* larvae died in HZM, HZZ and HCA whereas 97.33% and 98.67% mortality of the larvae were recorded in HZS and HZOR, respectively.

The KD and mortality activity were not observed in the control group for 24 h exposure.

The effective concentrations and exposure time of HZOR, HZM, HZS, HZZ and HCA to kill the *Ae*. *aegypti* larvae were 300 mg/L after 24 h; 200 mg/L after 24 h; 300 mg/L after 24 h; 300 mg/L after 6 h; and 300 mg/L after 24 h, respectively. Therefore, among the five Zingiberaceae species tested, HZZ showed the most effective in killing the *Ae. aegypti* larvae.

The results of GLZ analysis showed that the percentage mortality of *Ae*. *aegypti* larvae were significantly different within the hexane extracts ($F= 1430.43$, $df= 4$, $p<0.05$), the exposure time ($F= 4717.28$, $df= 3$, $p<0.05$) and the concentrations ($F= 4516.01$, $df= 3$, $p<0.05$). The interactions effect also differ significantly ($F= 1154.33$, $df= 36$, $p<0.05$). LSD Post Hoc test showed that all the five Zingiberaceae species were significantly different from each other and the highest percentage mortality of *Ae*. *aegypti* larvae was recorded in HZZ (Figure 4.4).





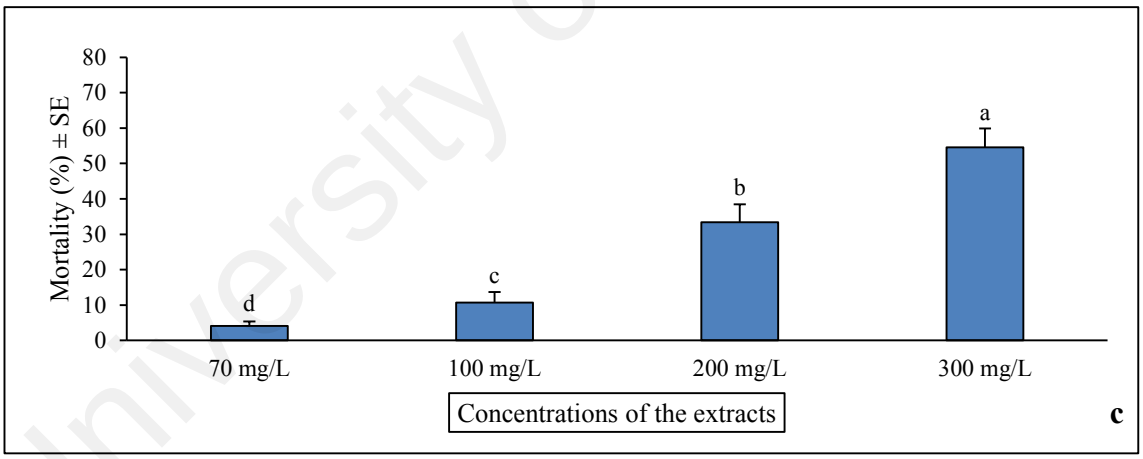


Figure 4.4: The mean percentage mortality of *Ae. aegypti* larvae after exposure to the hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA). Means with *same letters above bars* indicate no significant difference ($p<0.05$) (**a** different Zingiberaceae species, **b** different exposure time and **c** different concentrations)

4.3.3 Evaluation of the hexane extracts of five Zingiberaceae species against *Cx. quinquefasciatus* larvae

The larvicidal activity of *Cx*. *quinquefasciatus* after being tested with HZOR, HZM, HZS, HZZ and HCA at different exposure time and concentrations were shown in Figure 4.5. At 1 h exposure, all the *Cx*. *quinquefasciatus* larvae survived when exposed to HZOR, HZM and HZS at all the concentrations, but 2.67% KD activity of *Cx*. *quinquefasciatus* was observed in HZZ at concentration of 200 mg/L and 1.33% KD activity was observed in HCA at 100 mg/L, 200 mg/L and 300 mg/L.

At 3 h exposure, less than 5.00% KD activity was observed in all the hexane extracts at 70 mg/L and 100 mg/L. At 200 mg/L, KD activity of *Cx*. *quinquefasciatus* larvae in HZOR and HZS were much lower (6.67% and 4.00%) than in HZZ and HCA (17.33% and 24.00%) while there was no KD activity in HZM. At 300 mg/L, high KD activity of *Cx*. *quinquefasciatus* larvae was reported in HZOR and HZZ (93.33% and 94.67%); moderate KD activity was reported in HZM and HZS; and low KD activity was recorded in HCA (20.00%).

At 6 h exposure, the KD activity of *Cx*. *quinquefasciatus* larvae was relatively low (25.00%) at the concentrations of 70 mg/L and 100 mg/L in all the hexane extracts of five Zingiberaceae species. At 200 mg/L, HZM and HZS exhibited low KD activity (4.00% and 17.33%) while HZOR and HCA exhibited moderate KD activity (52.00% and 69.33%) and HZZ exhibited high (93.33%) KD activity. At 300 mg/L, the highest KD activity of the larvae was recorded in HZOR and HZZ (100.00%) compared to HZS (94.67%), HCA (97.33%) and HZM (72.00%).

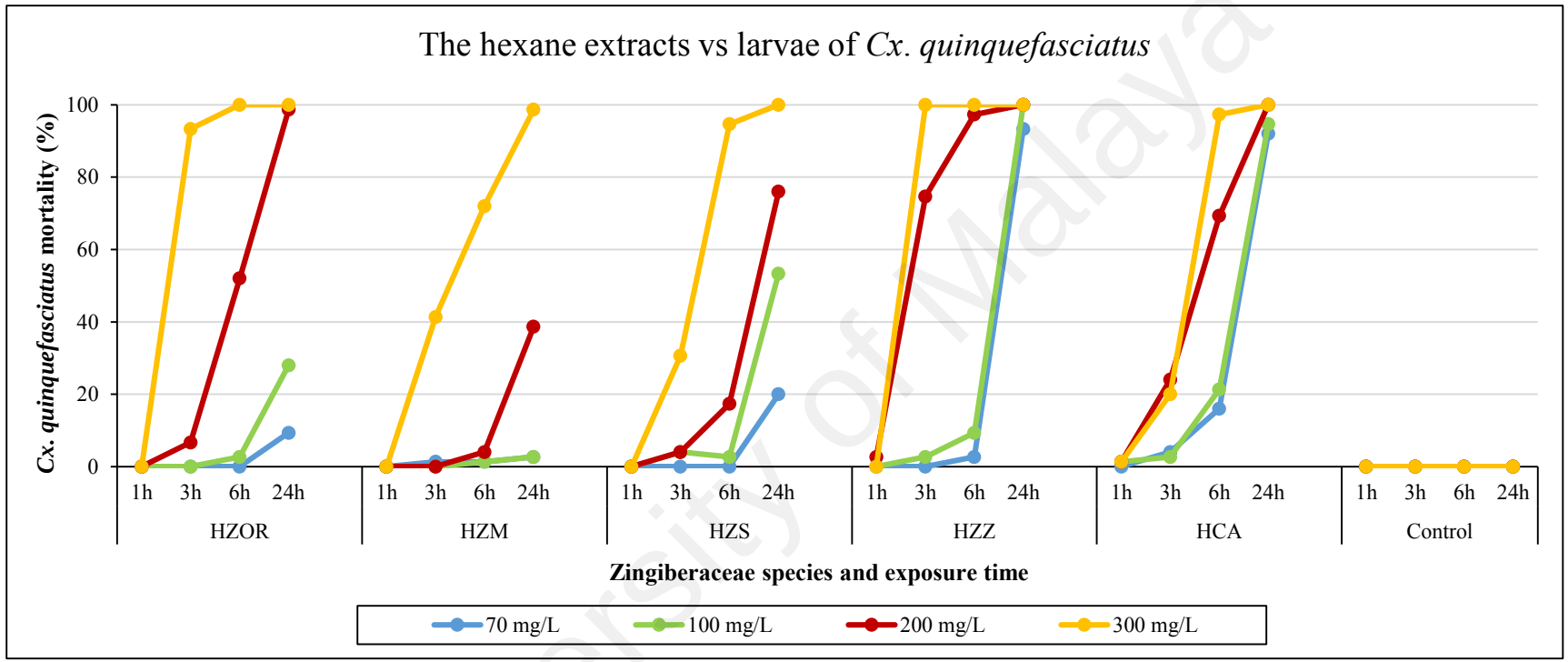
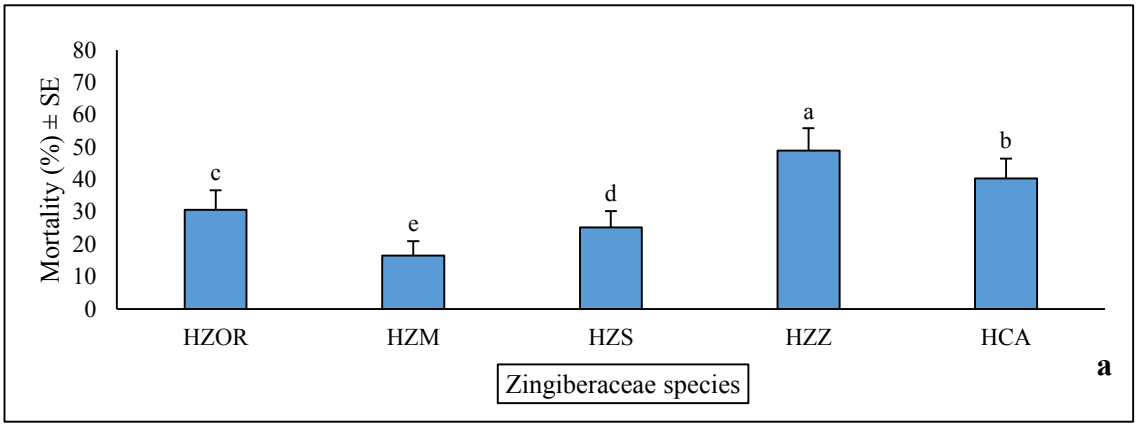


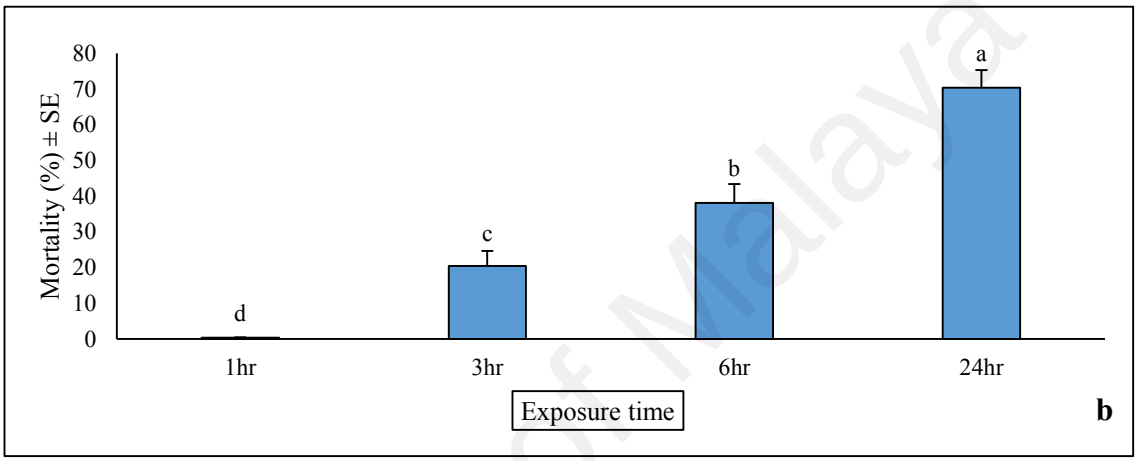
Figure 4.5: The larvicidal activity of hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA) against larvae of *Cx. quinquefasciatus* at different exposure time with various concentrations

After 24 h exposure, both HZZ and HCA showed high mortality of *Cx*. *quinquefasciatus* larvae even at the lowest concentration (70 mg/L) while the rest hexane extracts showed less than 20.00% mortality at the same concentration. At 100 mg/L, HZZ and HCA also exhibited high mortality of *Cx*. *quinquefasciatus* (100.00% and 92.00%); HZS showed moderate mortality (53.33%) while relatively low to very low mortality of the larvae was observed in HZOR (28.00%) and HZM (2.67%). More than 95.00% mortality of *Cx*. *quinquefasciatus* larvae was recorded at 200 mg/L in HZOR, HZZ and HCA, whereas 76.00% mortality was detected in HZS and only 38.67% mortality was reported in HZM. However, at 300 mg/L, all the hexane extracts exhibited high mortality ranging from 94.67% to 100.00%. There was no KD and mortality activity of the control group throughout the exposure time.

According to GLZ analysis, the Zingiberaceae species ($F= 990.52$, $df= 4$, $p<0.05$), the exposure time ($F= 5089.92$, $df= 3$, $p<0.05$), the concentrations ($F= 3092.42$, $df= 3$, $p<0.05$), and their interactions ($F= 1101.41$, $df= 36$, $p<0.05$), were significantly different. Results from LSD post hoc test showed that all the Zingiberaceae species, exposure time and concentrations significantly affected the percentage mortality of the *Cx. quinquefasciatus* larvae. The highest concentration (300 mg/L) and the longest exposure time (24 h) exhibited high mortality of the larvae (Figure 4.6).

The effective concentrations and exposure time of the hexane extracts of five Zingiberaceae species tested in killing the *Cx*. *quinquefasciatus* larvae were HZOR (at 300 mg/L after 6 h exposure), HZM (at 300 mg/L after 24 h exposure), HZS (at 300 mg/L after 24 h exposure), HZZ (at 300 mg/L after 3 h exposure) and HCA (at 200 mg/L after 24 h exposure). Therefore, among these hexane extracts, HZZ was noted as the most effective extract compared to others in killing the *Cx*. *quinquefasciatus* larvae.





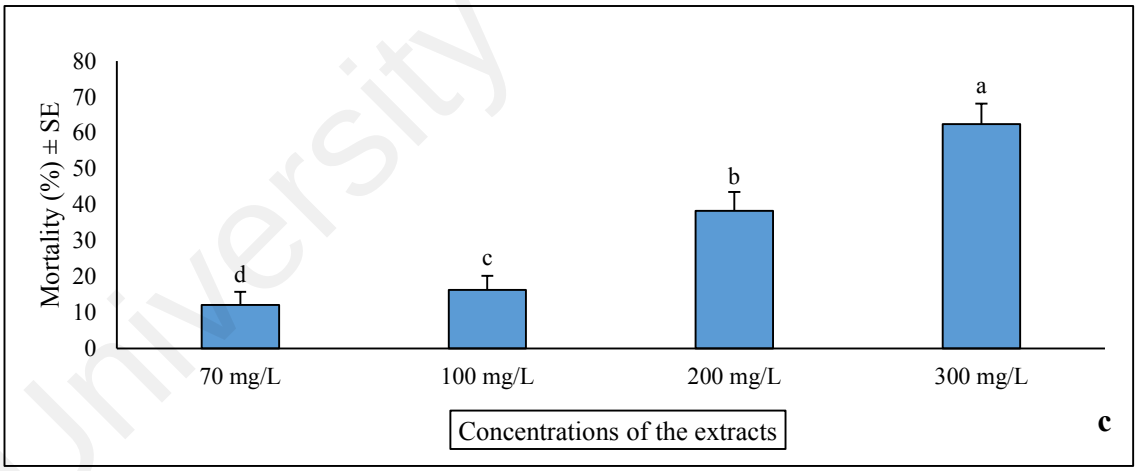


Figure 4.6: The mean percentage mortality of *Cx. quinquefasciatus* larvae after exposure to the hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA). Means with *same letters above bars* indicate no significant difference ($p<0.05$) (**a** different Zingiberaceae species, **b** different exposure time and **c** different concentrations)

4.3.4 Evaluation of the dichloromethane extracts of five Zingiberaceae species against *Ae. albopictus* larvae

Figure 4.7 showed the larvicidal activity of *Ae. albopictus* after being exposed to the dichloromethane extracts of *Z. officinale* var. *rubrum* (DZOR), *Z. montanum* (DZM), *Z. spectabile* (DZS), *Z. zerumbet* (DZZ) and *C. aeruginosa* (DCA) at various concentrations and different exposure time. The results showed that there was no KD activity observed in all the dichloromethane extracts at all the concentrations after being exposed for 1 h. After 3 h exposure, DZOR did not exhibit any KD activity of *Ae. albopictus* larvae at all the concentrations, whereas the rest of the four Zingiberaceae dichloromethane extracts exhibited KD activity of the larvae but only at 200 mg/L and 300 mg/L. At 300 mg/L, DCA showed higher KD activity of *Ae. albopictus* larvae (76.00%) than in DZM (25.33%), DZS (16.00%) and DZZ (21.33%) while at 200 mg/L, the KD activity of the larvae was recorded only less than 10.00% in DZM (1.33%), DZS (2.67%), DZZ (2.67%) and DCA (6.67%).

After being exposed for 6 h, high KD activity of *Ae. albopictus* larvae was reported in DCA (84.00%) at 300 mg/L while moderate KD activity of the larvae was shown in DZM, DZS and DZZ (38.67%, 45.33% and 52.00%, respectively). DZOR as is the case of 3 h exposure, also did not show any KD activity of *Ae. albopictus* larvae at all the concentrations after 6 h exposure. Lower to much lower percentage of KD activity of *Ae. albopictus* larvae was recorded in DZM at 100 mg/L and 200 mg/L (1.33% and 5.33%), in DZS at 70 mg/L, 100 mg/L and 200 mg/L (1.33%, 2.67% and 4.00%, respectively), in DZZ at 200 mg/L (10.67%) and in DCA at 200 mg/L (18.67%).

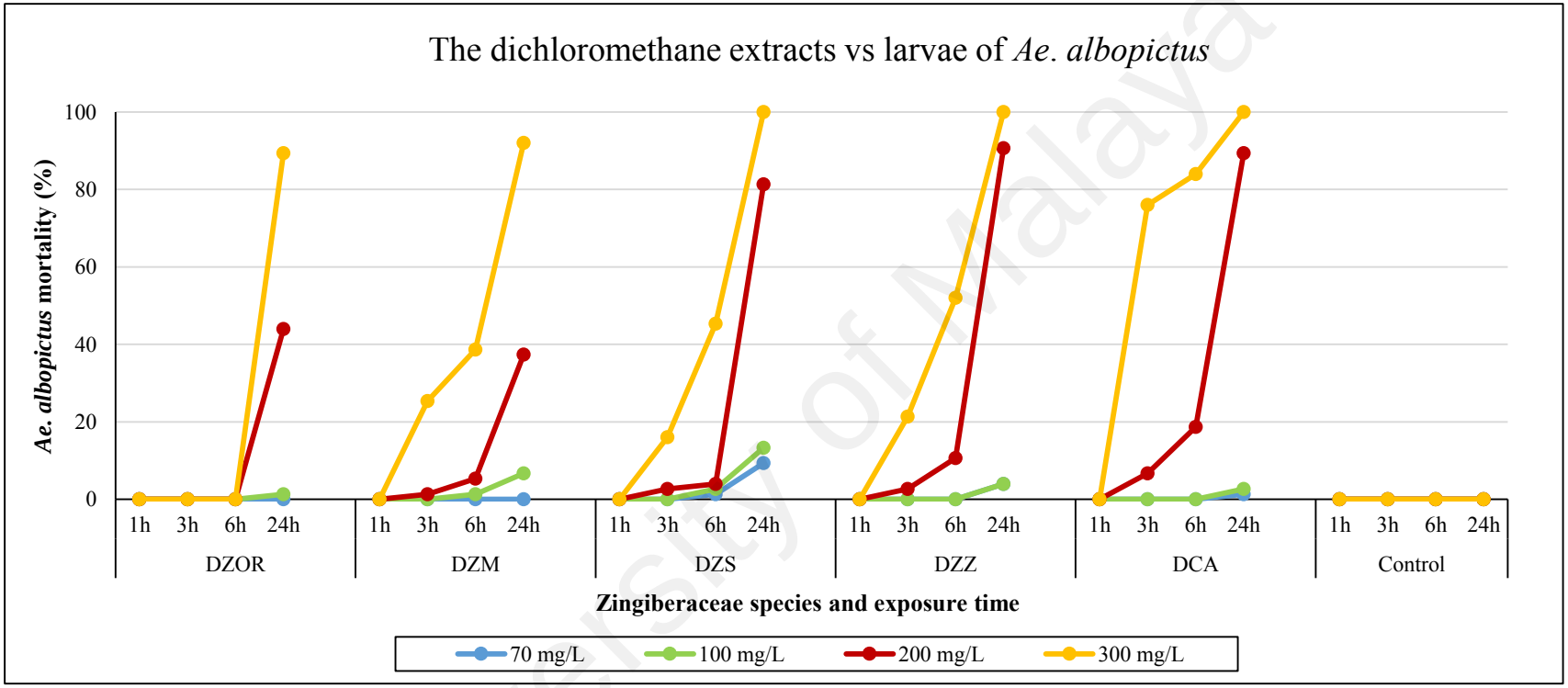
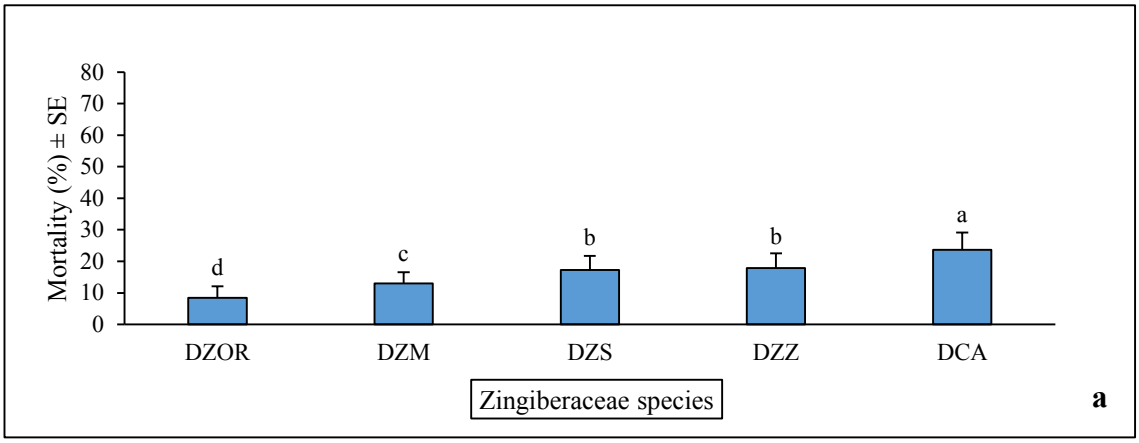


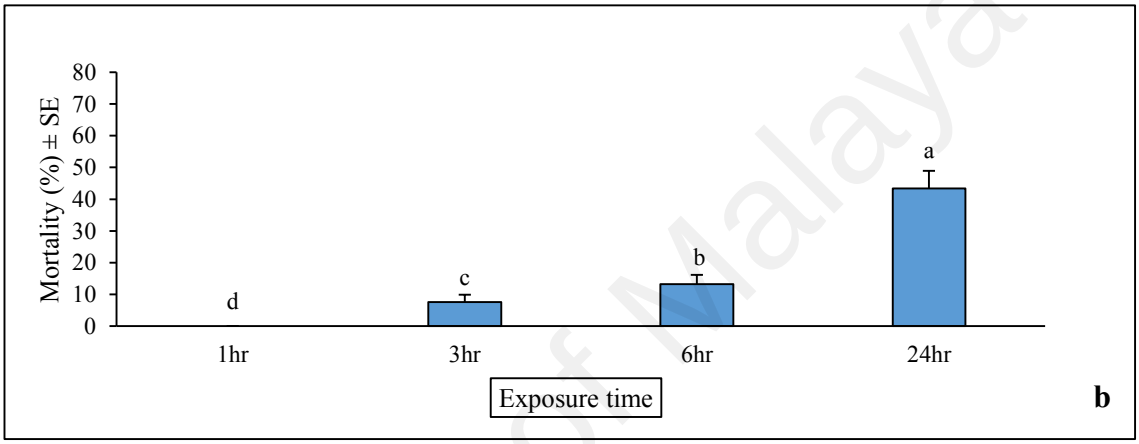
Figure 4.7: The larvicidal activity of dichloromethane extracts of *Z. officinale* var. *rubrum* (DZOR), *Z. montanum* (DZM), *Z. spectabile* (DZS), *Z. zerumbet* (DZZ) and *C. aeruginosa* (DCA) against larvae of *Ae. albopictus* at different exposure time with various concentrations

At the end of 24 h exposure, low percentage mortality of *Ae*. *albopictus* larvae (1.33% - 13.33%) was detected at the lower concentrations (70 mg/L and 100 mg/L) in all the dichloromethane extracts. At 200 mg/L, both DZOR and DZM showed moderate percentage mortality of the larvae (44.00% and 37.33%) whereas DZS, DZZ and DCA showed high percentage mortality (81.33%, 90.67% and 89.33%, respectively). At 300 mg/L, all *Ae*. *albopictus* larvae died after being tested with DZS, DZZ and DCA while 89.33% and 92.00% mortality of the larvae were observed in DZOR and DZM.

In the control group, no KD and mortality was observed from the first hour of exposure until 24 h exposure.

According to the GLZ ANOVA analysis, percentage mortality of *Ae*. *albopictus* larvae when exposed to the dichloromethane extracts of five Zingiberaceae species were significantly different ($F= 201.75$, $df= 4$, $p<0.05$). The percentage mortality was also different within the time periods ($F= 2094.74$, $df= 3$, $p<0.05$) and the concentrations ($F= 2185.44$, $df= 3$, $p<0.05$). The interactions among those three variables were also significant ($F= 362.38$, $df= 36$, $p<0.05$). The highest percentage mortality of *Ae. albopictus* larvae in dichloromethane extracts was recorded in DCA, followed by DZZ and DZS, DZM and the lowest was DZOR. The percentage mortality of the larvae was significantly different between exposure time (Figure 4.8).





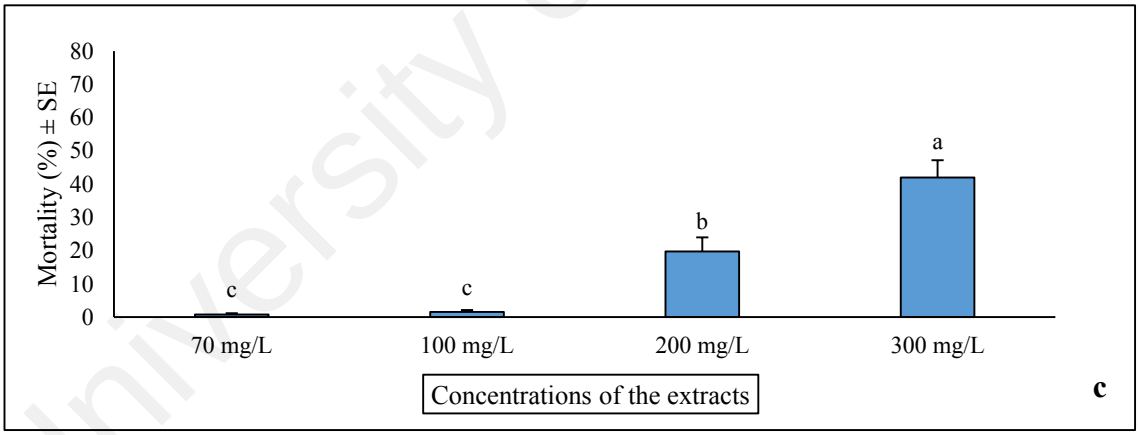


Figure 4.8: The mean percentage mortality of *Ae. albopictus* larvae after exposure to the dichloromethane extracts of *Z. officinale* var. *rubrum* (DZOR), *Z. montanum* (DZM), *Z. spectabile* (DZS), *Z. zerumbet* (DZZ) and *C. aeruginosa* (DCA). Means with *same letters above bars* indicate no significant difference ($p<0.05$) (**a** different Zingiberaceae species, **b** different exposure time and **c** different concentrations)

Overall, all dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* were effective to control *Ae. albopictus* larvae at 300 mg/L after 24 h exposure. However, since the highest mean of the percentage mortality of *Ae. albopictus* was obtained by DCA, therefore DCA was considered as the most effective to control the larvae.

4.3.5 Evaluation of the dichloromethane extracts of five Zingiberaceae species against *Ae. aegypti* larvae

The larvicidal activity of *Ae. aegypti* against DZOR, DZM, DZS, DZZ and DCA are presented in Figure 4.9. The results revealed that at 1 h exposure, all the dichloromethane extracts did not exhibit any KD activity at all the concentrations, except for DZZ at the concentration of 300 mg/L (14.67%). After 3 h exposure, all dichloromethane extracts did not exhibit any KD activity at 70 mg/L, but at 100 mg/L and 200 mg/L, low KD activity of *Ae. aegypti* larvae were observed only in DZM (1.33% and 1.33%, respectively) and DZZ (1.33% and 6.67%, respectively). At 300 mg/L, moderate KD activity was recorded in DZM (44.00%), DZS (48.00%), DZZ (69.33%) and DCA (65.33%).

At 6 h exposure, only DZOR did not show KD activity of *Ae. aegypti* larvae at all the concentrations whereas the rest of the dichloromethane extracts did not exhibit KD activity at the concentration of 70 mg/L. At 100 mg/L, only DZM and DZZ showed KD activity (1.33%); at 200 mg/L, DZM (4.00%), DZS (10.67%) and DCA (8.00%) showed lower KD activity than DZZ (30.67%). Meanwhile, at 300 mg/L, both DZM (62.67%) and DZS (69.33%) showed moderate KD activity while both DZZ and DCA showed high KD activity (88.00% and 85.33%, respectively).

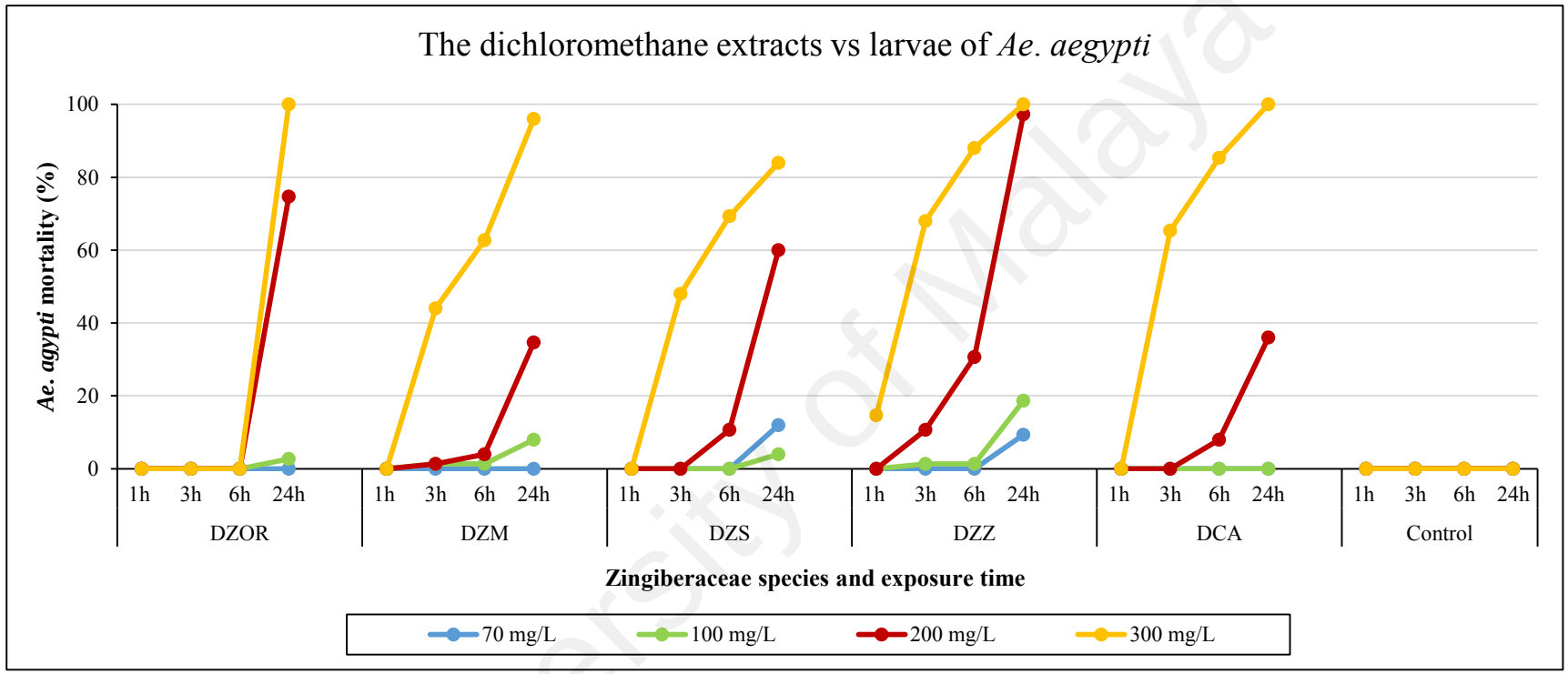


Figure 4.9: The larvicidal activity of dichloromethane extracts of *Z. officinale* var. *rubrum* (DZOR), *Z. montanum* (DZM), *Z. spectabile* (DZS), *Z. zerumbet* (DZZ) and *C. aeruginosa* (DCA) against larvae of *Ae. aegypti* at different exposure time with various concentrations

After 24 h exposure, the mortality of *Ae*. *aegypti* larvae in all the dichloromethane extracts ranged from 0.00% to 18.67% at 70 mg/L and 100 mg/L. At 200 mg/L, the highest percentage mortality of *Ae*. *aegypti* larvae was reported in DZZ (97.33%), followed by DZOR and DZS which showed moderate percentage mortality (74.67% and 60.00%) whereas low percentage mortality of *Ae*. *aegypti* larvae was recorded in DZM (34.67%) and DCA (36.00%). At 300 mg/L, 100.00% mortality of *Ae*. *aegypti* larvae was recorded in DZOR, DZZ and DCA while DZM and DZS showed slightly lower percentage mortality (96.00% and 84.00%).

There was no KD and mortality activity observed in the control group for all the exposure time.

Following the GLZ ANOVA, the results showed that there was significant difference on the means of five Zingiberaceae species ($F= 278.92$, $df= 4$, $p<0.05$), the exposure time ($F= 2206.01$, $df= 3$, $p<0.05$) and the concentrations ($F= 4031.92$, $df= 3$, $p<0.05$). The interactions between the Zingiberaceae species, exposure time and concentrations were also significant ($F= 519.81$, $df= 36$, $p<0.05$). LSD post hoc test (Figure 4.10) showed that the percentage mortality of *Ae*. *aegypti* larvae did not differ significantly between DZS and DCA. In addition, at the concentration of 300 mg/L, high percentage mortality of *Ae*. *aegypti* larvae was observed as compared to the concentrations of 70 mg/L, 100 mg/L and 200 mg/L.

The DZOR, DZM, DZS and DCA were effective in killing the *Ae*. *aegypti* larvae at 300 mg/L after 24 h exposure, while DZZ was effective in killing the *Ae*. *aegypti* larvae at 200 mg/L after 24 h. Therefore, DZZ was considered as the potent extract in killing the *Ae*. *aegypti* larvae.

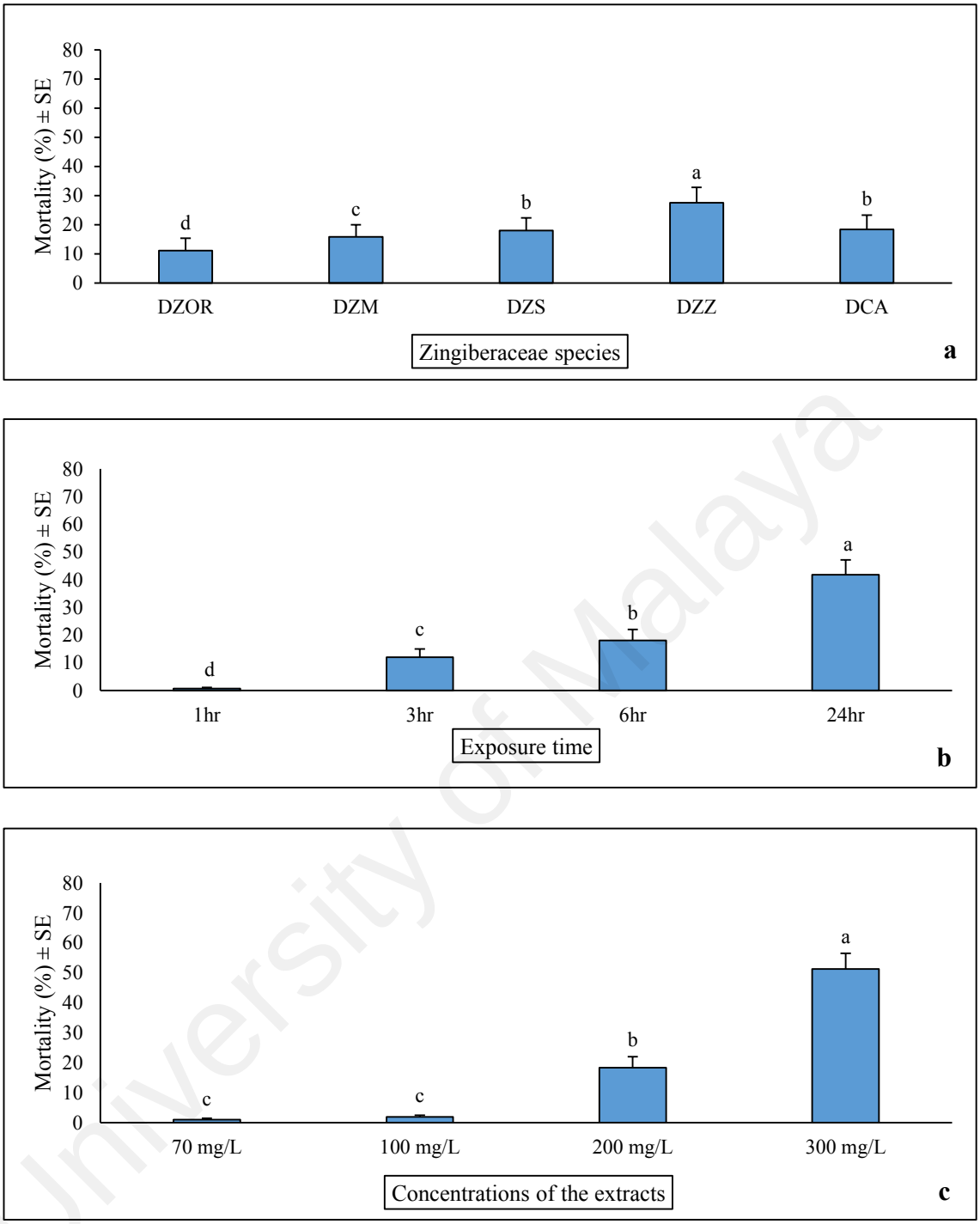


Figure 4.10: The mean percentage mortality of *Ae. aegypti* larvae after exposure to the dichloromethane extracts of *Z. officinale* var. *rubrum* (DZOR), *Z. montanum* (DZM), *Z. spectabile* (DZS), *Z. zerumbet* (DZZ) and *C. aeruginosa* (DCA). Means with *same letters above bars* indicate no significant difference ($p<0.05$) (**a** different Zingiberaceae species, **b** different exposure time and **c** different concentrations)

4.3.6 Evaluation of the dichloromethane extracts of five Zingiberaceae species against *Cx. quinquefasciatus* larvae

Figure 4.11 shows the results of larvicidal activity of *Cx. quinquefasciatus* in DZOR, DZM, DZS, DZZ, and DCA at different exposure time and concentrations. At 1 h exposure, all the *Cx. quinquefasciatus* larvae survived in all dichloromethane extracts at 70 mg/L, 100 mg/L, 200 mg/L and 300 mg/L. At 3 h exposure, no KD activity of *Cx. quinquefasciatus* larvae was recorded at 70 mg/L but only DCA exhibited KD activity (4.00%) at 100 mg/L. At 200 mg/L, moderate KD activity was detected only in DZZ and DCA (36.00% and 44.00%, respectively). At 300 mg/L, KD activity of *Cx. quinquefasciatus* larvae in DZZ, DZOR, DZM and DZS ranged from 57.33% - 5.33% but all *Cx. quinquefasciatus* larvae died in DCA.

After 6 h exposure, less than 10.00% KD activity of *Cx. quinquefasciatus* larvae were recorded at the concentrations of 70 mg/L and 100 mg/L in all dichloromethane extracts. At 200 mg/L, DZZ exhibited high KD activity (85.33%), DCA exhibited moderate KD activity (56.00%) whereas DZOR, DZM and DZS exhibited low KD activity (10.67%, 2.67% and 2.67%, respectively). At 300 mg/L, DCA showed relatively high KD activity (98.67%) while DZM and DZS showed moderate KD activity (42.67% and 46.67%) but all larvae died in DZOR and DZZ.

At 24 h exposure, both DZZ and DCA exhibited high mortality of the *Cx. quinquefasciatus* larvae at 70 mg/L with 85.33% and 81.33%, respectively compared to DZOR (12.00%), DZM (1.33%) and DZS (2.67%). At the concentrations of 70 mg/L and 100 mg/L, high mortality of *Cx. quinquefasciatus* larvae was reported in DZZ (94.67%) and DCA (85.33%) but at 100 mg/L, the larvae was not much affected by DZOR (9.33%), DZS (1.33%) and none died in DZM. Similarly, at 200 mg/L, all *Cx. quinquefasciatus* larvae died in DZZ and DCA but showed moderate mortality in DZOR (48.00%) and DZS (37.33%) and relatively low mortality in DZM (17.30%). Likewise at 300 mg/L,

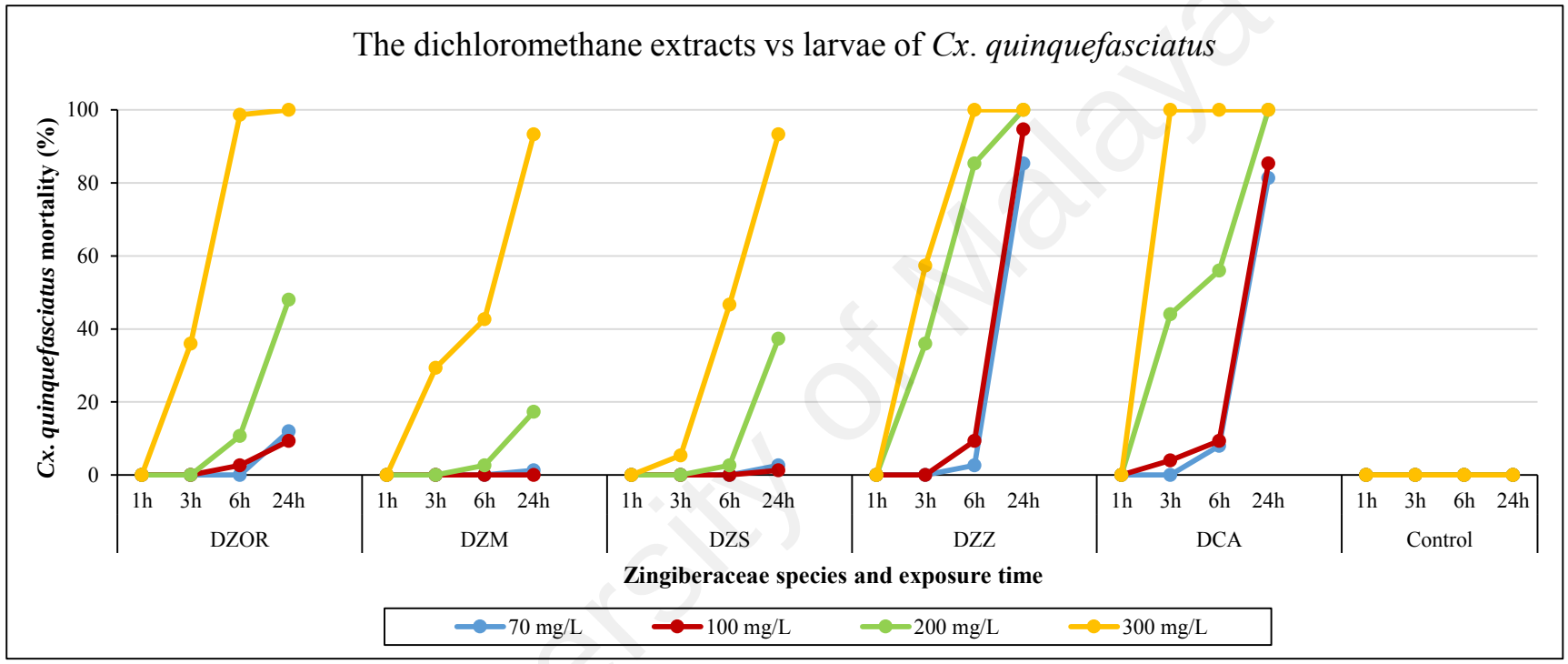


Figure 4.11: The larvicidal activity of dichloromethane extracts of *Z. officinale* var. *rubrum* (DZOR), *Z. montanum* (DZM), *Z. spectabile* (DZS), *Z. zerumbet* (DZZ) and *C. aeruginosa* (DCA) against larvae of *Cx. quinquefasciatus* at different exposure time with various concentrations

all *Cx. quinquefasciatus* larvae died in DZOR, DZZ and DCA while 93.33% mortality was observed in DZM and DZS.

There was no KD and mortality activity for the control group observed from the first hour of exposure until the end of 24 h exposure time.

Generalized Linear Model (GLZ) was used and the results revealed that the percentage mortality of the *Cx*. *quinquefasciatus* larvae was significantly affected by the different Zingiberaceae species ($F= 1321.05$, $df= 4$, $p<0.05$), different exposure time ($F= 3055.82$, $df= 3$, $p<0.05$), different concentrations ($F= 2256.27$, $df= 3$, $p<0.05$) and the interactions ($F= 929.37$, $df= 36$, $p<0.05$). Mean comparison in post hoc test among DZM and DZS as well as DZZ and DCA did not differ significantly in the percentage mortality of *Cx*. *quinquefasciatus* larvae. Exposure time was significantly different and the 24 h exposure time exhibited the highest percentage mortality of the *Cx*. *quinquefasciatus* larvae (Figure 4.12).

Overall, the effectiveness of the concentrations and exposure time of the dichloromethane extracts of five Zingiberaceae species tested in killing the *Cx*. *quinquefasciatus* larvae were as follows: in DZOR was recorded at 300 mg/L after 6 h exposure; in DZM and DZS were recorded at 300 mg/L after 24 h exposure; in DZZ was detected at 300 mg/L after 6 h; and in DCA was recorded at 300 mg/L after 3 h exposure. Thus, among these five Zingiberaceae species tested, DCA was more effective in killing the *Cx*. *quinquefasciatus* larvae compared to the other dichloromethane extracts.

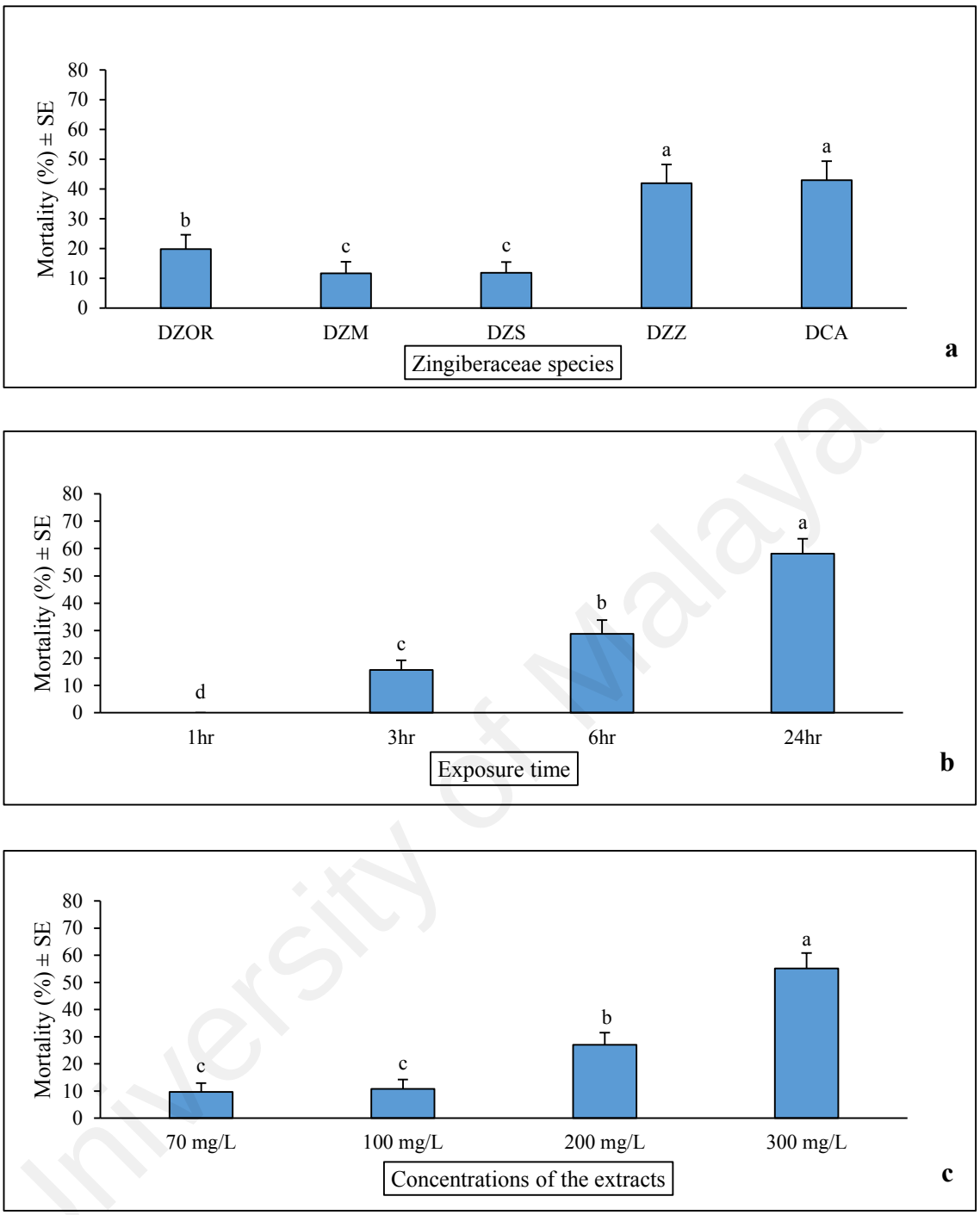


Figure 4.12: The mean percentage mortality of *Cx. quinquefasciatus* larvae after exposure to the dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet*, and *C. aeruginosa*. Means with *same letters above bars* indicate no significant difference ($p<0.05$) (**a** different Zingiberaceae species, **b** different exposure time and **c** different concentrations)

4.3.7 The most effective Zingiberaceae extracts for larvicidal activity against the three mosquito species

In conclusion, the best Zingiberaceae species to kill the larvae of *Ae*. *albopictus* were HZOR (300 mg/L, 6 h exposure) and DCA (300 mg/L, 24 h exposure). The most effective Zingiberaceae extracts to control the larvae of *Ae*. *aegypti* were HZZ (300 mg/L, 24 h exposure) and DZZ (200 mg/L, 6 h exposure). Meanwhile, the best plant candidates to kill the larvae of *Cx. quinquefasciatus* were HZZ (at 300 mg/L, 3 h exposure) and DCA (at 300 mg/L, 3 h exposure). Therefore, in terms of exposure time, among the three mosquito species, the larvae of *Cx*. *quinquefasciatus* was more susceptible to the hexane and dichloromethane extracts than the larvae of *Aedes* species in the shorter time.

4.4 LETHAL CONCENTRATIONS (LC_{50} & LC_{90}) OF THE HEXANE AND DICHLOROMETHANE EXTRACTS OF FIVE ZINGIBERACEAE SPECIES AGAINST *AE. ALBOPICTUS*, *AE. AEGYPTI* AND *CX. QUINQUEFASCIATUS* LARVAE

Table 4.5 and Table 4.6 presented the LC_{50} and LC_{90} values of the hexane and dichloromethane extracts of five Zingiberaceae species tested against the three mosquitoes after 24 h exposure. Classification of LC_{50} studies of plant extracts on mosquitoes by Komalamisra *et al*. (2005) stated that the larvicidal activity of plants are categorized as effective when LC_{50} value is below 750 mg/L, moderate when it is 50 mg/L to 100 mg/L, and high when it is lower than 50 mg/L. Hence, with reference to Komalamisra *et al*. (2005), all the hexane and dichloromethane extracts of five Zingiberaceae species from this study are considered effective ($LC_{50} < 750$ mg/L) against all the three mosquitoes. From this study, there were four extracts were categorized as high that is HZZ, DZZ, HCA and DCA with LC_{50} values of 49.28 mg/L, 30.15 mg/L,

Table 4.5: Lethal concentration values of the hexane extracts of five Zingiberaceae species against the larvae of three mosquito species

Plant species	Mosquito species	LC_{50} (mg/L)	95% Confidence limits (mg/L)		LC_{90} (mg/L)	Slope ± SE	χ^2	r
			Lower	Upper				
Z. officinale var. *rubrum*	*Ae. albopictus*	96.86	84.07	111.18	168.65	6.57 ± 2.15	12.00	0.91
	Ae. aegypti	120.60	112.03	130.29	187.85	8.63 ± 1.98	9.26	0.95
	Cx. quinquefasciatus	130.58	53.72	313.62	213.81	8.57 ± 2.25	12.00	0.94
Z. montanum	*Ae. albopictus*	99.04	87.39	114.87	153.77	7.37 ± 1.89	9.26	0.94
	Ae. aegypti	84.95	72.42	97.26	134.85	6.23 ± 1.96	12.00	0.91
	Cx. quinquefasciatus	176.35	73.11	288.51	275.97	8.20 ± 2.43	9.00	0.92
Z. spectabile	*Ae. albopictus*	93.51	78.34	108.84	168.65	6.30 ± 1.43	6.00	0.95
	Ae. aegypti	155.93	129.29	300.91	315.79	7.27 ± 1.68	12.00	0.95
	Cx. quinquefasciatus	107.78	87.90	128.31	228.83	6.57 ± 0.43	9.26	0.99
Z. zerumbet	*Ae. albopictus*	106.57	99.35	115.35	162.77	7.97 ± 2.34	12.00	0.92
	Ae. aegypti	82.05	70.93	92.79	121.05	5.87 ± 1.70	12.00	0.93
	Cx. quinquefasciatus	49.28	40.71	58.59	83.87	0.50 ± 0.29	9.26	0.78
C. aeruginosa	*Ae. albopictus*	159.15	133.93	189.91	259.26	8.57 ± 2.20	12.00	0.94
	Ae. aegypti	219.86	209.64	338.13	246.92	7.87 ± 3.52	12.00	0.85
	Cx. quinquefasciatus	21.94	0.23	41.38	66.61	0.73 ± 0.18	12.00	0.95

LC_{50}, lethal concentration that kills 50% of the exposed larvae; LC_{90}, lethal concentration that kills 90% of the exposed larvae
95% CL, 95% confidence limits; χ^2, chi-square value; r, correlation coefficient value

21.94 mg/L and 42.47 mg/L, respectively, which were effective to control the larvae of *Cx*. *quinquefasciatus*. In this respect, there are five extracts which are considered moderate, that is HZOR (LC_{50}= 96.86 mg/L), HZM (LC_{50}= 99.04 mg/L), HZS (LC_{50}= 93.51 mg/L) which were effective against the larvae of *Ae*. *albopictus* and HZM (84.95 mg/L) and HZZ (82.05 mg/L) were effective against the larvae of *Ae*. *aegypti*. However, on the overall, the hexane extracts appeared to be more effective than the dichloromethane extracts. Except for DZZ and DCA as mention above, the rest of the dichloromethane extracts are effective against all the mosquito species at higher concentrations ranging from 119.37 mg/L – 208.46 mg/L (Table 4.6).

Table 4.6: Lethal concentration values of the dichloromethane extracts of five Zingiberaceae species against the larvae of three mosquito species

Plant species	Mosquito species	LC_{50} (mg/L)	95% Confidence limits (mg/L)		LC_{90} (mg/L)	Slope ± SE	χ^2	r
			Lower	Upper				
Z. officinale var. *rubrum*	*Ae. albopictus*	206.93	192.17	221.42	307.67	7.77 ± 1.87	9.00	0.95
	Ae. aegypti	166.47	154.34	177.96	229.00	9.30 ± 2.24	12.00	0.95
	Cx. quinquefasciatus	166.59	69.75	332.29	320.97	7.57 ± 2.22	9.26	0.92
Z. montanum	*Ae. albopictus*	199.92	173.57	230.32	323.92	7.67 ± 1.90	12.00	0.94
	Ae. aegypti	197.41	169.76	229.93	316.94	7.87 ± 2.13	12.00	0.93
	Cx. quinquefasciatus	208.46	204.83	226.00	287.47	7.33 ± 3.14	12.00	0.86
Z. spectabile	*Ae. albopictus*	157.01	58.08	508.86	258.14	8.54 ± 1.89	12.00	0.95
	Ae. aegypti	177.62	146.84	220.65	367.49	6.80 ± 2.12	12.00	0.92
	Cx. quinquefasciatus	202.22	145.12	282.60	324.85	7.70 ± 2.29	12.00	0.92
Z. zerumbet	*Ae. albopictus*	141.21	120.86	166.43	202.88	9.37 ± 2.92	12.00	0.92
	Ae. aegypti	119.37	105.63	138.23	178.04	8.77 ± 2.58	12.00	0.92
	Cx. quinquefasciatus	30.15	5.71	46.64	82.62	1.23 ± 0.37	12.00	0.92
C. aeruginosa	*Ae. albopictus*	148.44	129.99	168.40	203.31	9.57 ± 2.87	12.00	0.92
	Ae. aegypti	139.02	106.73	182.92	222.33	8.40 ± 2.53	9.00	0.92
	Cx. quinquefasciatus	42.47	20.82	55.62	99.05	1.77 ± 0.48	9.00	0.94

LC_{50}, lethal concentration that kills 50% of the exposed larvae; LC_{90}, lethal concentration that kills 90% of the exposed larvae
95% CL, 95% confidence limits; χ^2, chi-square value; r, correlation coefficient value

4.5 ADULTICIDAL BIOASSAYS

The hexane and dichloromethane extracts of *Z. officinale* var. *rubrum* (HZOR and DZOR), *Z. montanum* (HZM and DZM), *Z. spectabile* (HZS and DZS), *Z. zerumbet* (HZZ and DZZ) and *C. aeruginosa* (HCA and DCA) were investigated for adulticidal activity against three mosquito species (*Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*). The concentration of 1000 mg/m^2 was applied in this experiment. There was no knockdown and mortality of the mosquitoes observed in the control group throughout the 24 h exposure time.

4.5.1 Adulticidal bioassays of the hexane and dichloromethane extracts of five Zingiberaceae species against *Ae. albopictus*

The results of adulticidal bioassays of the hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA) against *Ae. albopictus* at different exposure time are illustrated in Figure 4.13. The results revealed that *Ae. albopictus* was only resistant to HZM with 0.00% mortality for 24 h exposure. HZOR, HCA and HZZ showed low knockdown (KD) activity (2.22%) of *Ae. albopictus* after being exposed for 1 to 2 h. HCA (20.00%) exhibited higher KD activity at 3 h exposure than HZZ, HZS and HZOR with 8.89%, 8.89% and 2.22%, respectively. At the end of 24 h exposure, the highest mortality of *Ae. albopictus* was reported in HCA (37.78%), followed by HZS (24.44%), HZZ (20.00%) and HZOR (6.67%).

Statistical analysis showed a significant difference at 5% significance level between the exposure time and the percentage mortality of *Ae. albopictus* for each of the hexane extracts (HZOR, HZM, HZS, HZZ and HCA) with $F= 93.44$, $df= 12$, $p<0.05$. Therefore, among these hexane extracts, the most effective extract in killing the *Ae. albopictus* mosquito was HCA.

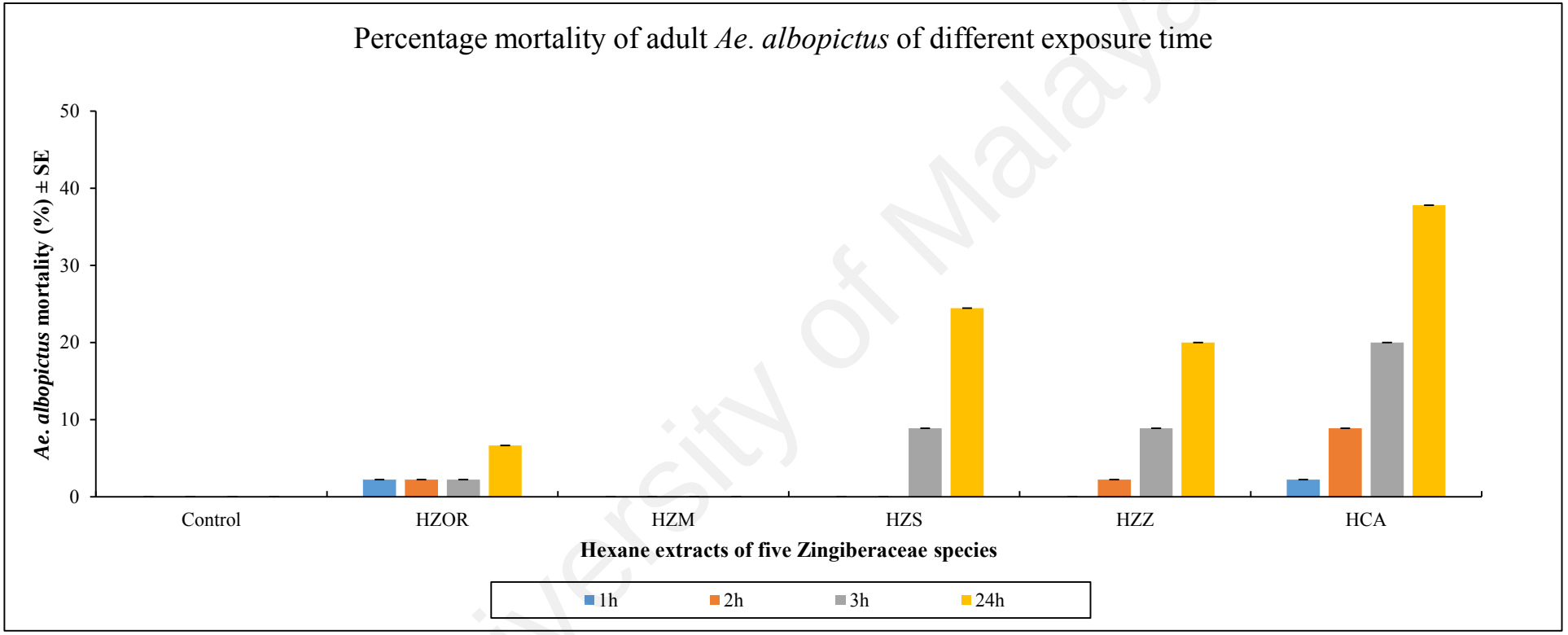


Figure 4.13: Influence of exposure time among the hexane extracts on the percentage mortality of adult *Ae. albopictus*

The regression equations (y= mx+c) and regression values (R^2) were reported in HZOR, HZS, HZZ and HCA. The R^2 values of HZOR, HZS, HZZ and HCA were 0.75, 0.70, 0.81 and 0.83, respectively. It showed that > 70% of the total mortality of *Ae. albopictus* was caused by duration of the exposure time (Figure 4.14).

Figure 4.15 showed the adulticidal activity of the dichloromethane extracts of *Z. officinale* var. *rubrum* (DZOR), *Z. montanum* (DZM), *Z. spectabile* (DZS), *Z. zerumbet* (DZZ) and *C. aeruginosa* (DCA) against *Ae. albopictus* at different exposure time. The results revealed that only DZS and DZZ showed 2.22% KD activity of *Ae. albopictus* after being exposed for 3 h. After 24 h exposure, the percentage mortality in DZS was higher (15.56%) than in DZZ (6.67%). Thus, DZS was considered to be more effective in killing *Ae. albopictus* than DZZ. However, the adults of *Ae. albopictus* were not affected at all by DZM even after 24 h exposure.

Statistical analysis showed a significant difference at 5% significance level between the exposure time and the percentage mortality of *Ae. albopictus* for each of the dichloromethane extracts (DZOR, DZM, DZS, DZZ and DCA) with F= 39.47, df= 12, $p<0.05$. The regression values were shown only in DZS and DZZ with values of 0.69 and 0.85 (Figure 4.16). It explained that 69% and 85% of the total mortality of *Ae. albopictus* was affected by different exposure time. Therefore, among the hexane and dichloromethane extracts, HCA induced high mortality of *Ae. albopictus* than the other extracts.

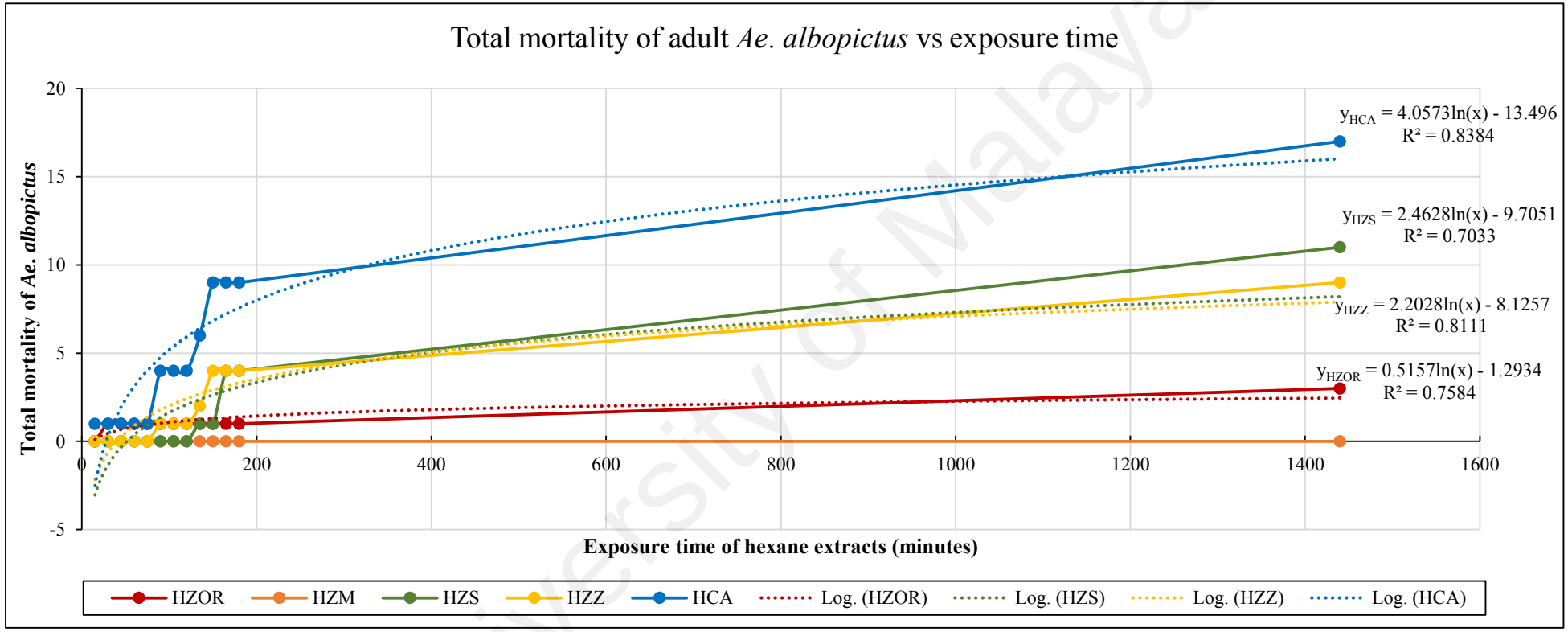


Figure 4.14: The regression analysis between total mortality of adult *Ae. albopictus* and the exposure time of the hexane extracts

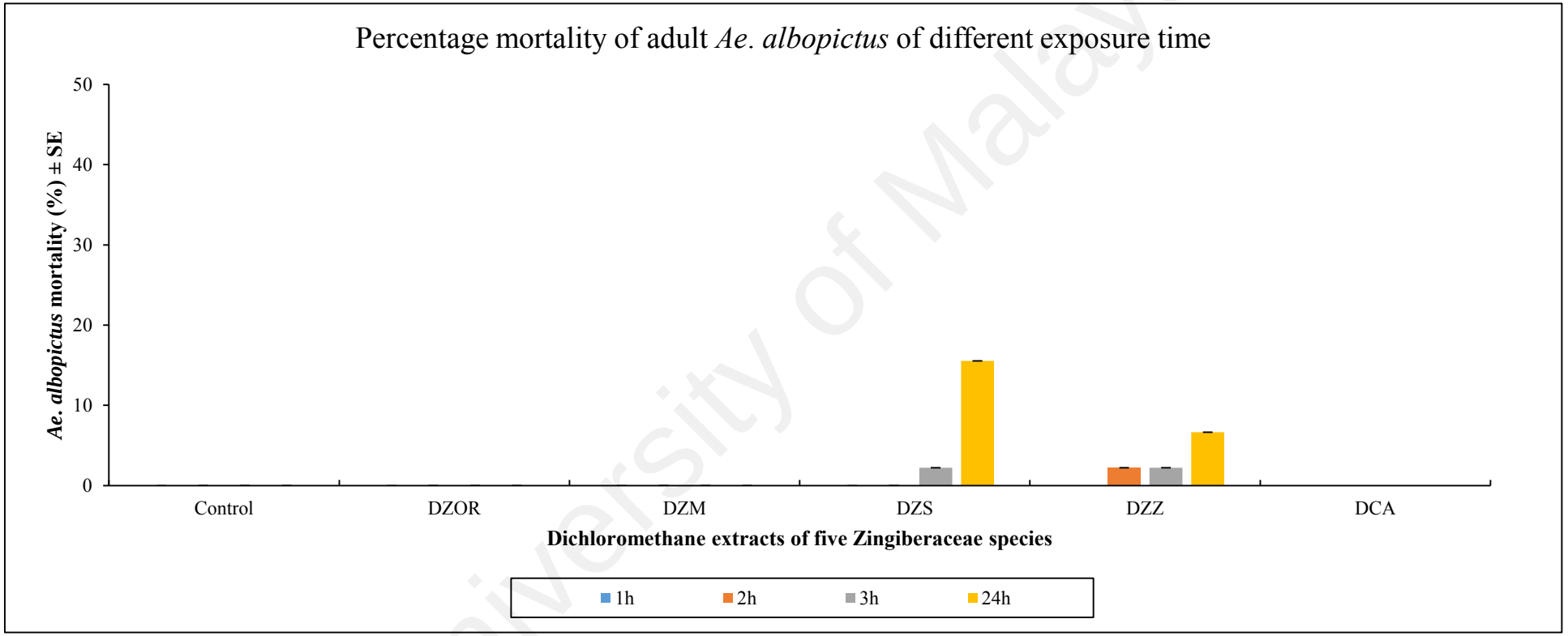


Figure 4.15: Influence of exposure time among the dichloromethane extracts on the percentage mortality of adult *Ae. albopictus*

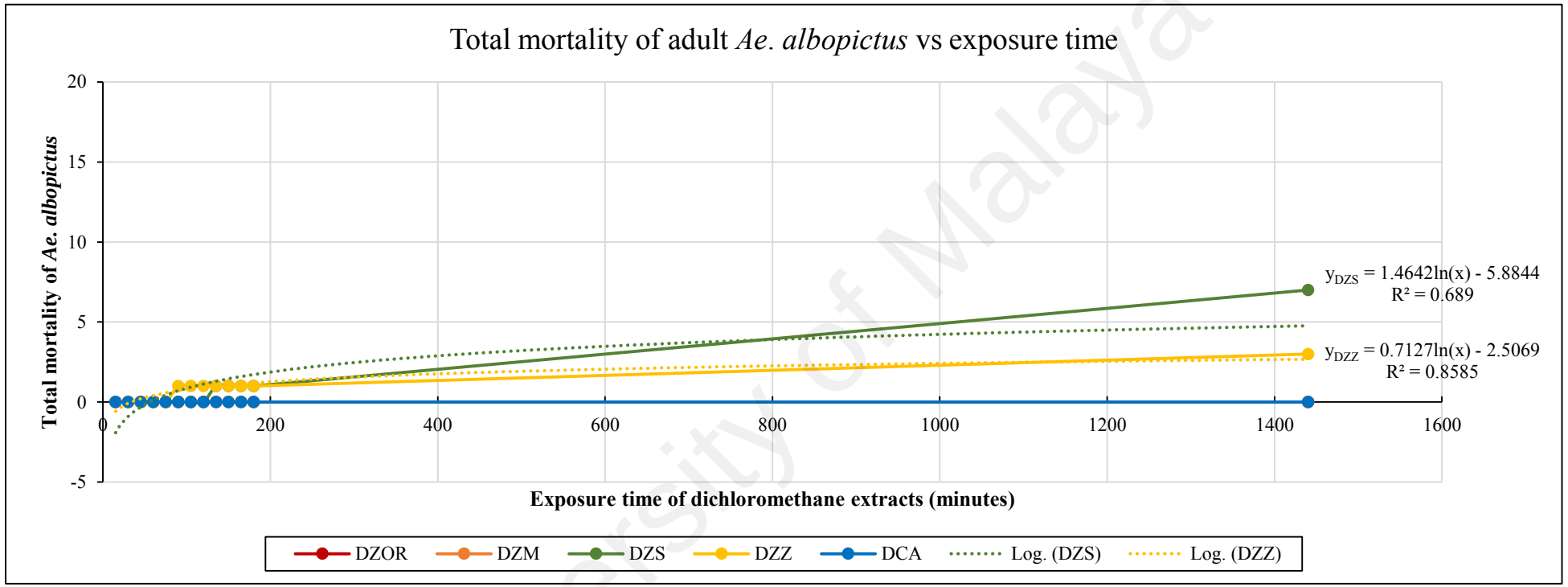


Figure 4.16: The regression analysis between total mortality of adult *Ae. albopictus* and the exposure time of the dichloromethane extracts

4.5.2 Adulticidal bioassays of the hexane and dichloromethane extracts of five Zingiberaceae species against *Ae. aegypti*

Figure 4.17 shows the adulticidal activity of the hexane extracts of *Z. officinale* var. *rubrum* (HZOR), *Z. montanum* (HZM), *Z. spectabile* (HZS), *Z. zerumbet* (HZZ) and *C. aeruginosa* (HCA) against *Ae. aegypti* at different exposure time. The results showed that there was no KD activity of *Ae. aegypti* observed in all the hexane extracts after 3 h exposure. After 24 h exposure, HZOR and HZS exhibited 2.22% mortality while HZZ exhibited 4.44%. However, the adults of *Ae. aegypti* were not affected at all by HZM even after 24 h exposure. Thus, among the hexane extracts, HZZ was found to be relatively effective in killing *Ae. aegypti*. There was a significant difference at 5% significance level between the exposure time and the percentage mortality of *Ae. aegypti* for each of the hexane extracts (HZOR, HZM, HZS, HZZ and HCA) with $F= 36.00$, $df= 12$, $p<0.05$. Figure 4.18 showed the regression values of total mortality of *Ae. aegypti* in HZOR, HZS and HZZ. These three hexane extracts had regression values of 0.56.

The adulticidal activity of the dichloromethane extracts of five Zingiberaceae species against *Ae. aegypti* at different exposure time are presented in Figure 4.19. The results revealed that KD activity was observed only in DZZ (2.22%) after 3 h exposure. After 24 h exposure, the highest percentage mortality was reported in DZOR (6.67%), followed by DCA (4.44%) and DZZ (2.22%). However, the adults of *Ae. aegypti* were not affected at all by DZM and DZS even after 24 h exposure. The regression values of DZOR and DCA ($R^2= 0.55$) were higher than DZZ ($R^2= 0.45$) (Figure 4.20). There was a significant difference between the exposure time and the percentage mortality of *Ae. aegypti* for each of the dichloromethane extracts (DZOR, DZM, DZS, DZZ and DCA) at 5% significance level ($F= 51.77$, $df= 12$, $p<0.05$). Therefore, among the hexane and dichloromethane extracts, DZOR appeared to be the most effective extract in killing *Ae. aegypti* compared to the others.

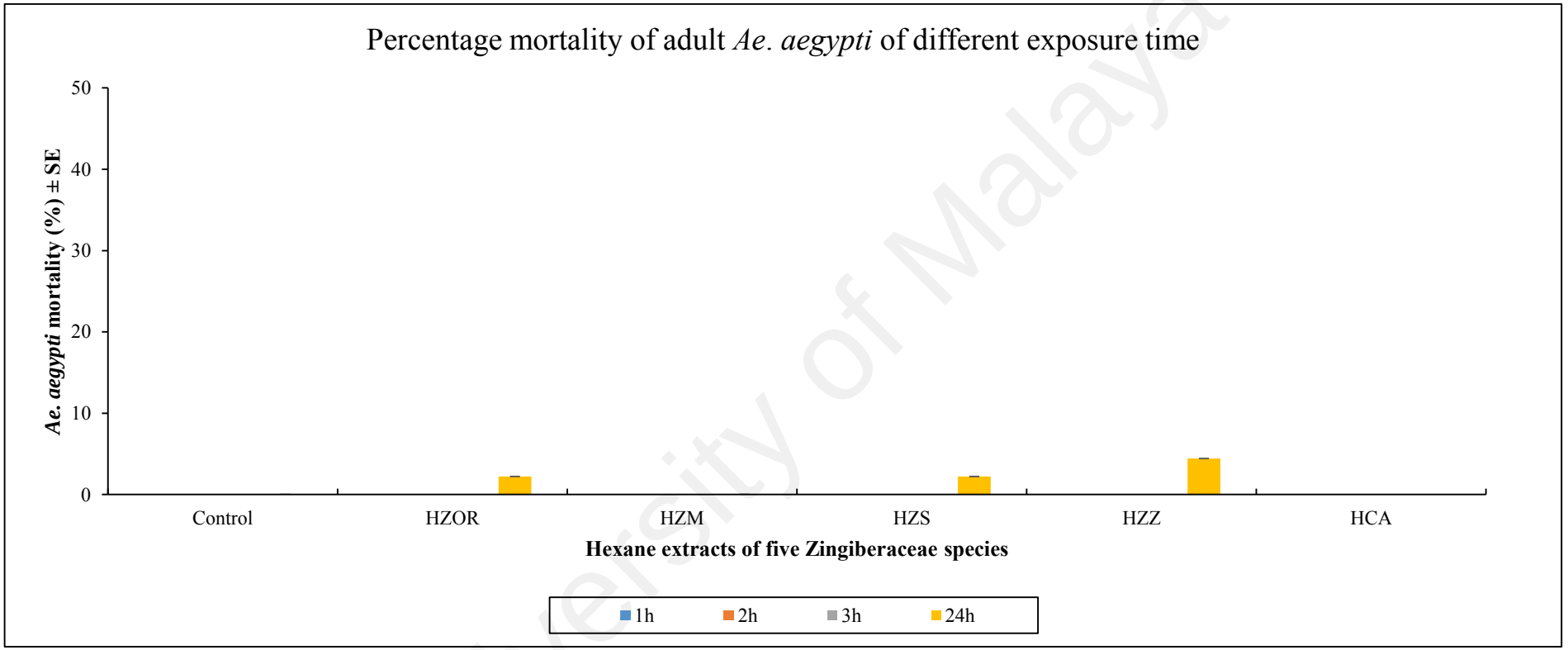


Figure 4.17: Influence of exposure time among the hexane extracts on the percentage mortality of adult *Ae. aegypti*

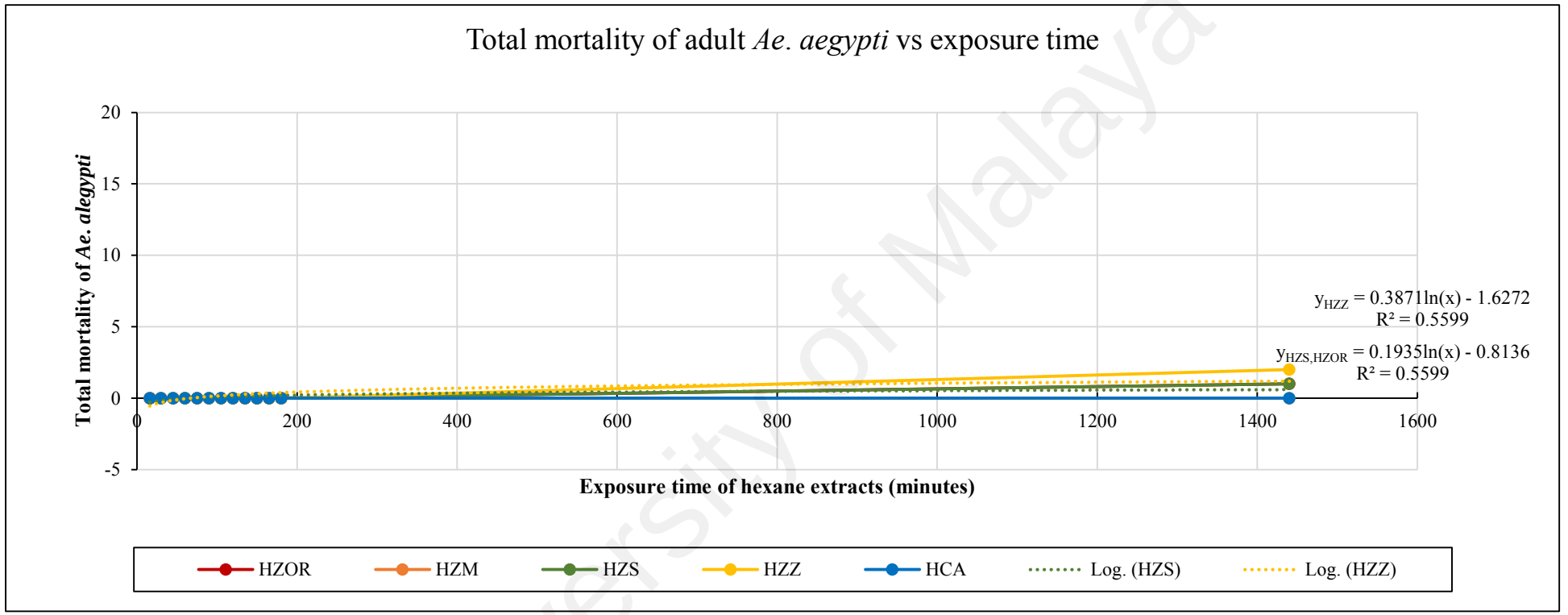


Figure 4.18: The regression analysis between total mortality of adult *Ae. aegypti* and the exposure time of the hexane extracts

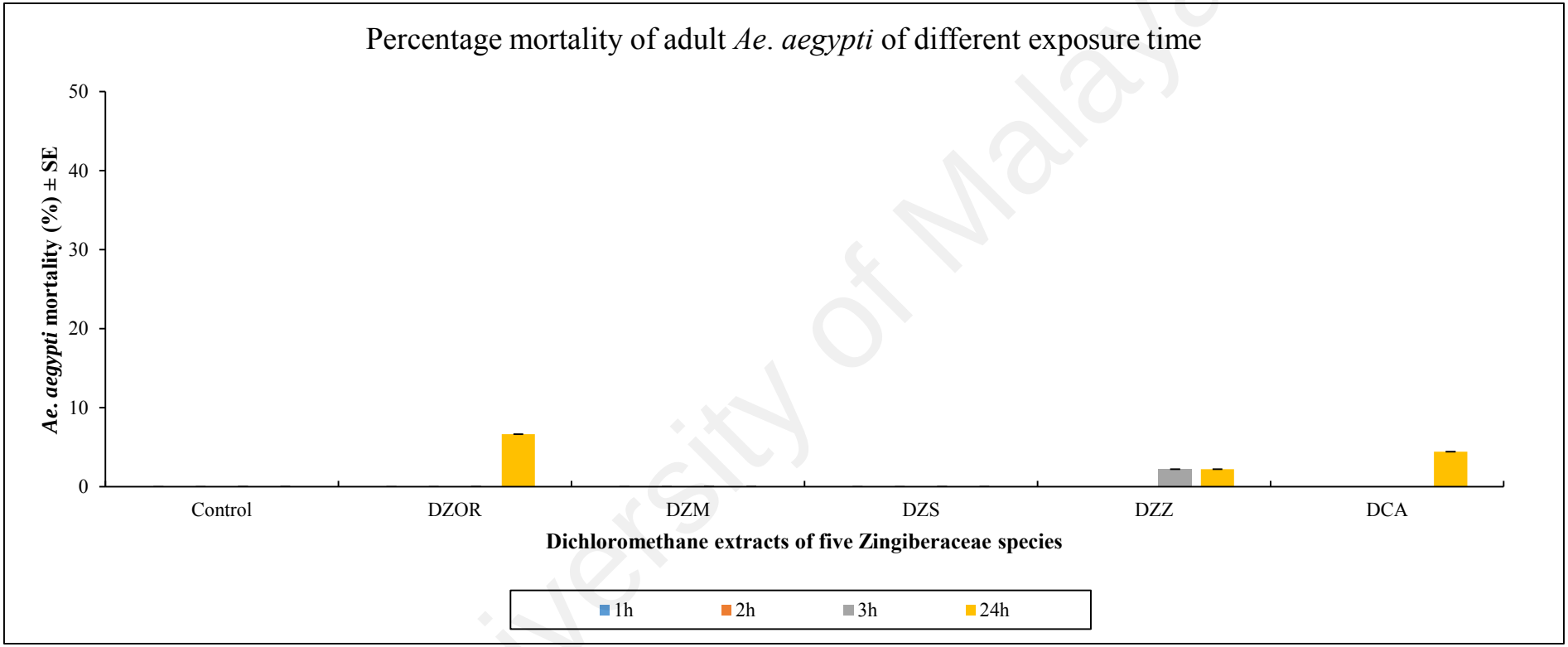


Figure 4.19: Influence of exposure time among the dichloromethane extracts on the percentage mortality of adult *Ae. aegypti*

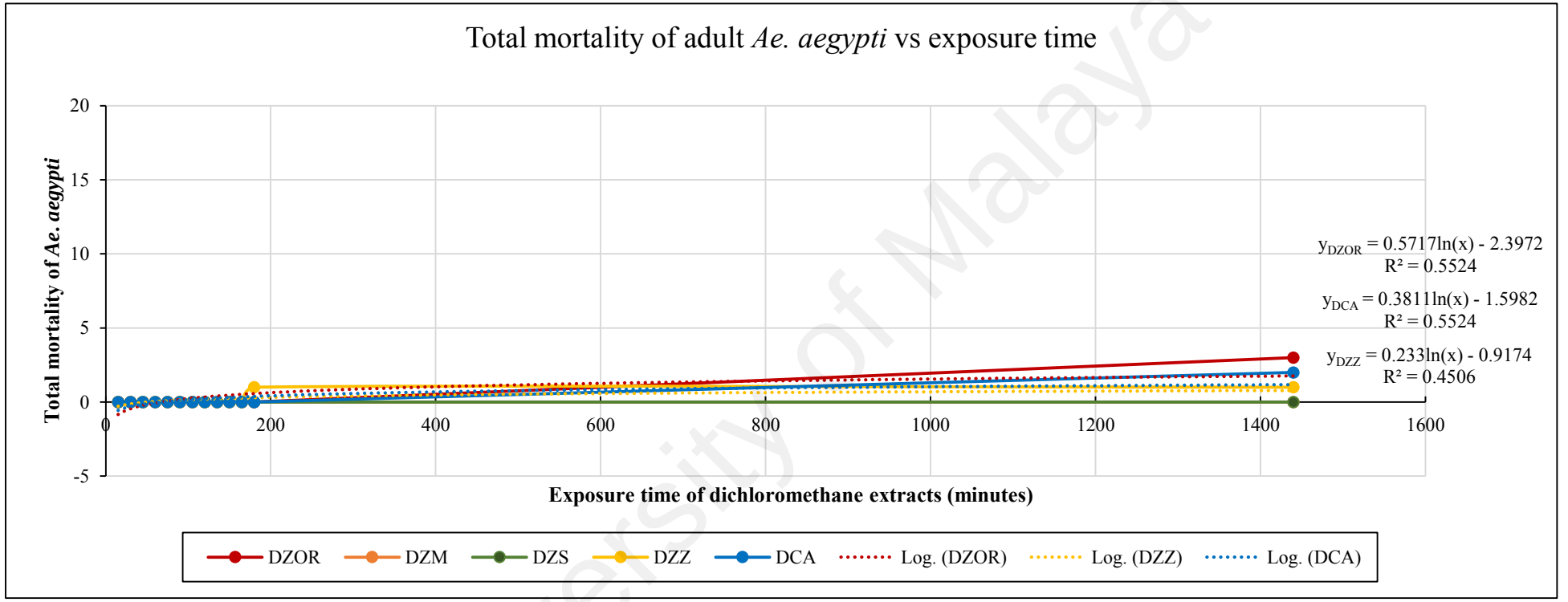


Figure 4.20: The regression analysis between total mortality of adult *Ae. aegypti* and the exposure time of the dichloromethane extracts

4.5.3 Adulticidal bioassays of the hexane and dichloromethane extracts of five Zingiberaceae species against *Cx. quinquefasciatus*

The results of adulticidal activity of the hexane and dichloromethane extracts of five plant species against *Cx*. *quinquefasciatus* are shown in Figure 4.21 and Figure 4.22. The most active extracts in killing *Cx*. *quinquefasciatus* were reported only in the hexane extract of *C*. *aeruginosa* (HCA) and the dichloromethane extract of *Z*. *zerumbet* (DZZ) with 2.22% mortality after 3 and 24 h exposure.

Both HCA and DZZ showed R^2= 0.44 (Figure 4.23 and Figure 4.24). There was no significant difference at 5% significance level between the exposure time and the percentage mortality of *Cx. quinquefasciatus* for each of the hexane and dichloromethane extracts ($F= 12.00$, $df= 12$, $p>0.05$).

The three mosquitoes tested were highly resistant to HZM and DZM but relatively susceptible to the other hexane and dichloromethane extracts. HCA was the most effective extract in killing *Ae*. *albopictus* whereas DZOR was the most effective against *Ae*. *aegypti*. Both HCA and DZZ were effective to control *Cx*. *quinquefasciatus*. In summary, the hexane and dichloromethane extracts of five Zingiberaceae species exhibited less than 40.00% mortality of *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus*.

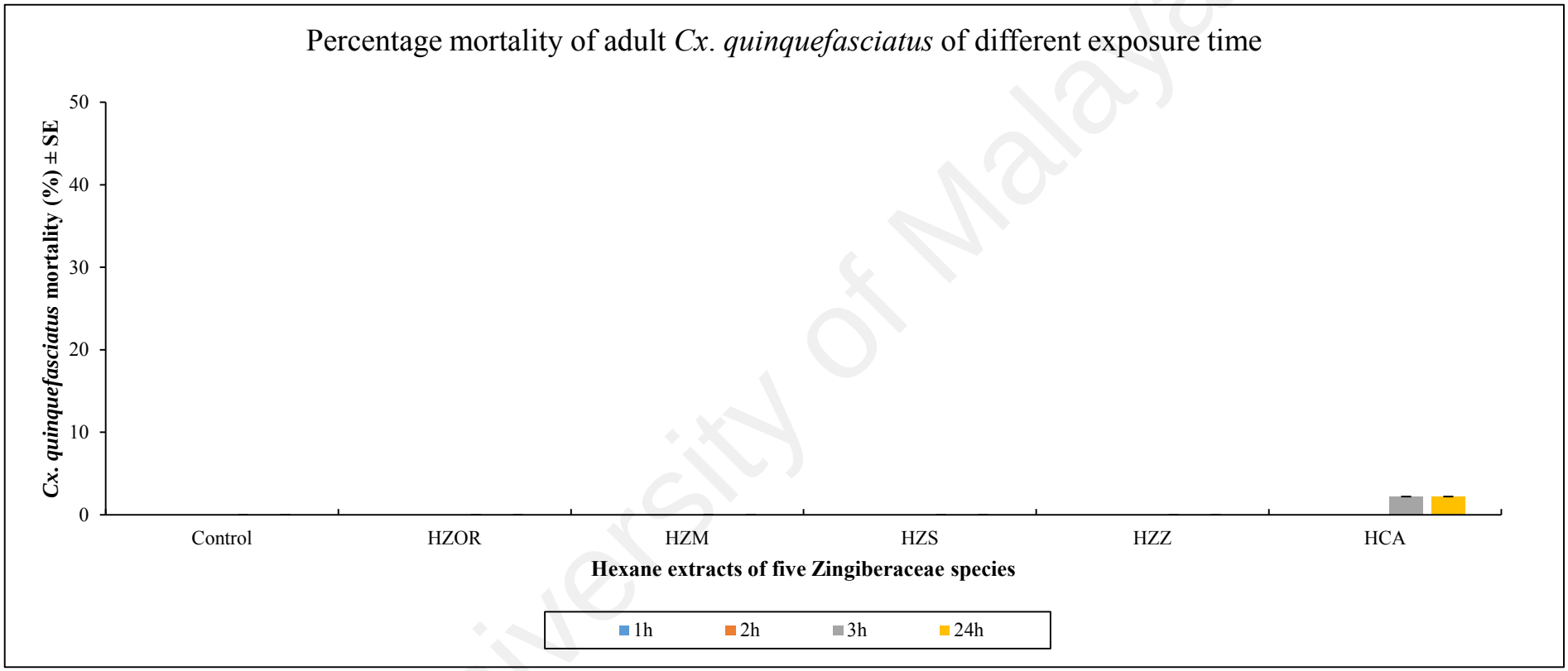


Figure 4.21: Influence of exposure time among the hexane extracts on the percentage mortality of adult *Cx. quinquefasciatus*

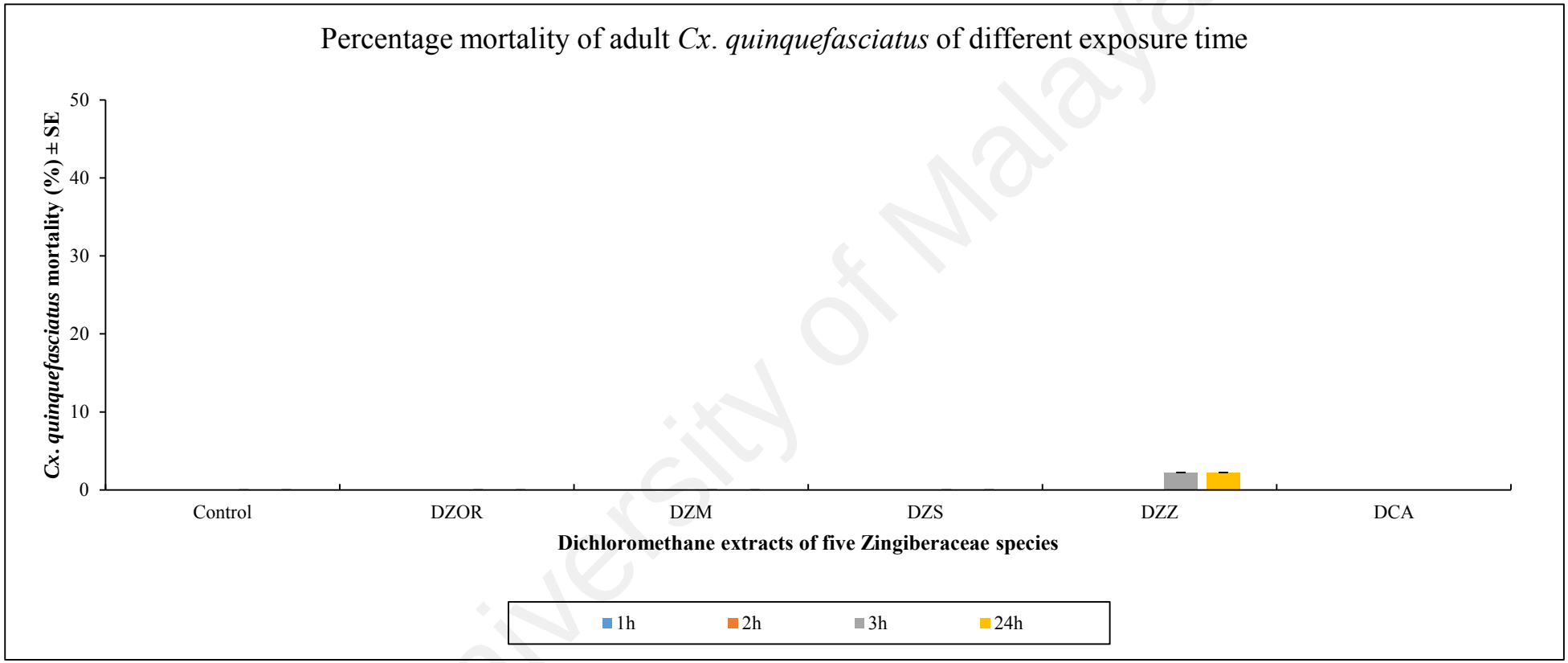


Figure 4.22: Influence of exposure time among the dichloromethane extracts on the percentage mortality of adult *Cx. quinquefasciatus*

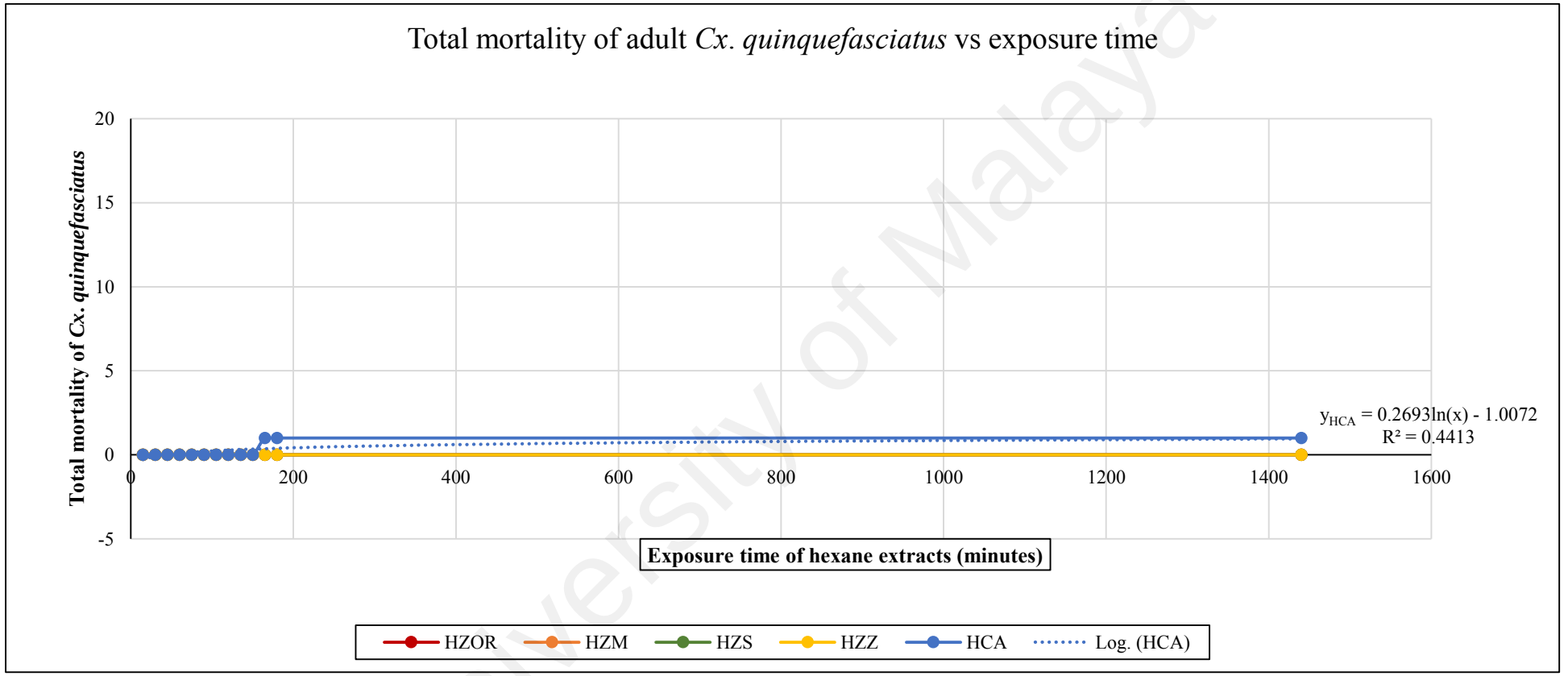


Figure 4.23: The regression analysis between total mortality of adult *Cx. quinquefasciatus* and the exposure time of the hexane extracts

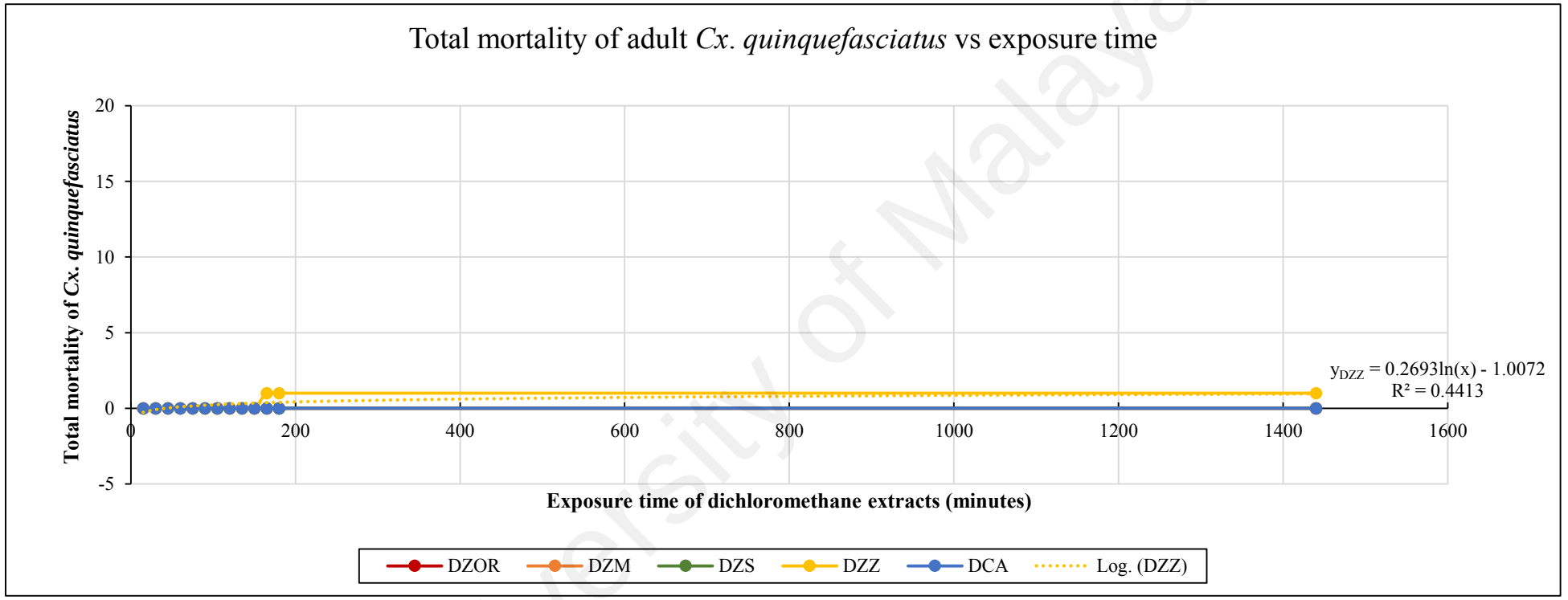


Figure 4.24: The regression analysis between total mortality of adult *Cx. quinquefasciatus* and the exposure time of the dichloromethane extracts

4.6 REPELLENCY, BLOOD-FEEDING INHIBITION AND MORTALITY ACTIVITY OF FIVE ZINGIBERACEAE SPECIES AGAINST *AE. ALBOPICTUS*, *AE. AEGYPTI* AND *CX. QUINQUEFASCIATUS*

4.6.1 Repellency activity of the hexane and dichloromethane extracts of five Zingiberaceae species against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*

a) Hexane extract of *Z. officinale* var. *rubrum*

The result for hexane extract of *Z. officinale* var. *rubrum* (HZOR) repellencies against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* at different exposure time is presented in Figure 4.25. The repellency of the three mosquitoes ranged from 90.67% - 100.00% after being exposed to HZOR for 30 minutes, 1 h and 3 h exposure. After being exposed to HZOR for 24 h, *Ae. albopictus* was less repelled (37.33%) compared to *Ae. aegypti* and *Cx. quinquefasciatus* (62.67% and 65.33%).

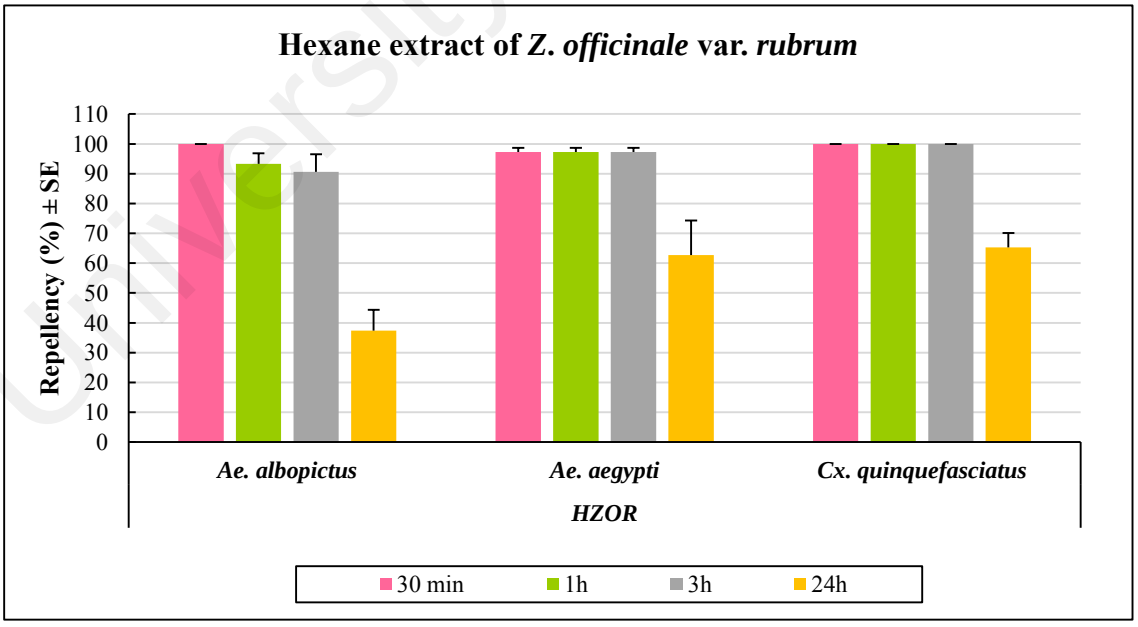


Figure 4.25: Repellency of the hexane extract of *Z. officinale* var. *rubrum* (HZOR) against the three adult mosquito species at different exposure time

b) Dichloromethane extract of *Z. officinale* var. *rubrum*

In the dichloromethane extract of *Z. officinale* var. *rubrum* (DZOR), *Ae. albopictus* and *Cx. quinquefasciatus* showed 100.00% repellency throughout the 3 exposure time (30 min, 1 h and 3 h) while *Ae. aegypti* showed relatively high repellency (77.33% - 88.00%). However, at the end of 24 h exposure, both *Aedes* species (46.67%) were less repelled than *Cx. quinquefasciatus* (53.33%) (Figure 4.26).

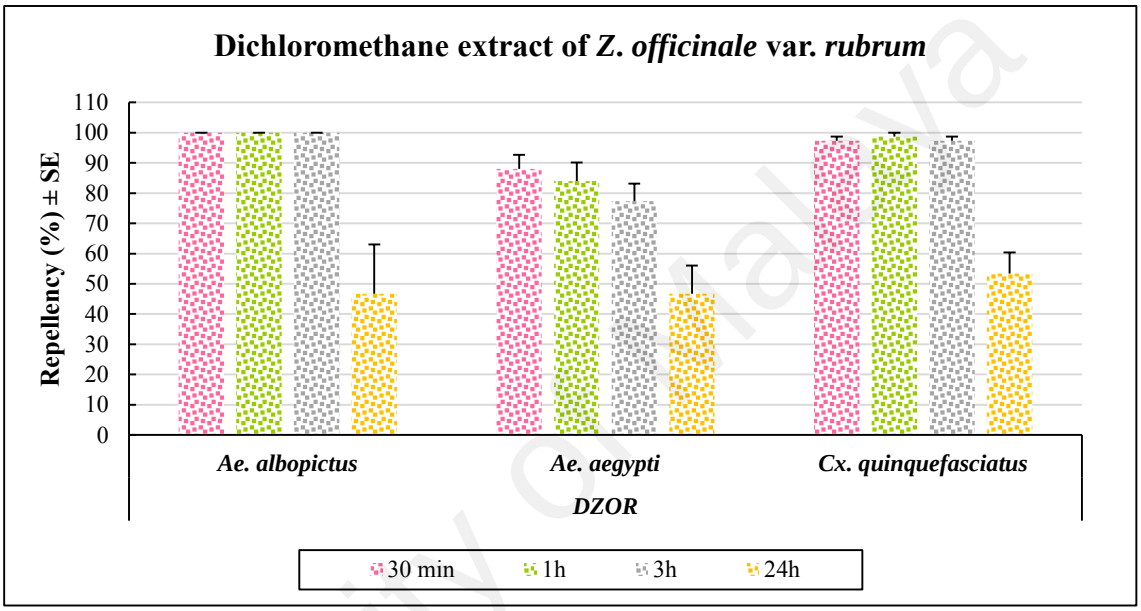


Figure 4.26: Repellency of the dichloromethane extract of *Z. officinale* var. *rubrum* (DZOR) against the three adult mosquito species at different exposure time

There were significant differences in the repellencies of the three mosquito species at different exposure time in HZOR ($F= 22.61$, $df= 3$, $p<0.05$) and DZOR ($F= 20.43$, $df= 3$, $p<0.05$). Therefore, HZOR was effective in repelling *Ae. aegypti* and *Cx. quinquefasciatus* while DZOR was effective in repelling *Ae. albopictus*.

c) Hexane and dichloromethane extracts of *Z. montanum*

Figure 4.27 and Figure 4.28 show the repellencies of the hexane and dichloromethane extracts of *Z. montanum* (HZM and DZM) against the three mosquito species at different exposure time. The results revealed that after 30 minutes, 1 h and 3 h exposure, both HZM and DZM exhibited > 85% repellency against *Ae. albopictus* (86.67% - 98.67%), *Ae.*

aegypti (97.33% - 98.67%) and *Cx. quinquefasciatus* (97.33% - 98.67%). However, after being exposed for 24 h, the repellency of the two extracts vary according to the mosquito species that is high for *Ae. aegypti* (89.33% and 85.33%), relatively high to moderate for *Cx. quinquefasciatus* (72.00% and 54.67%), and moderate for *Ae. albopictus* (49.33% and 46.67%).

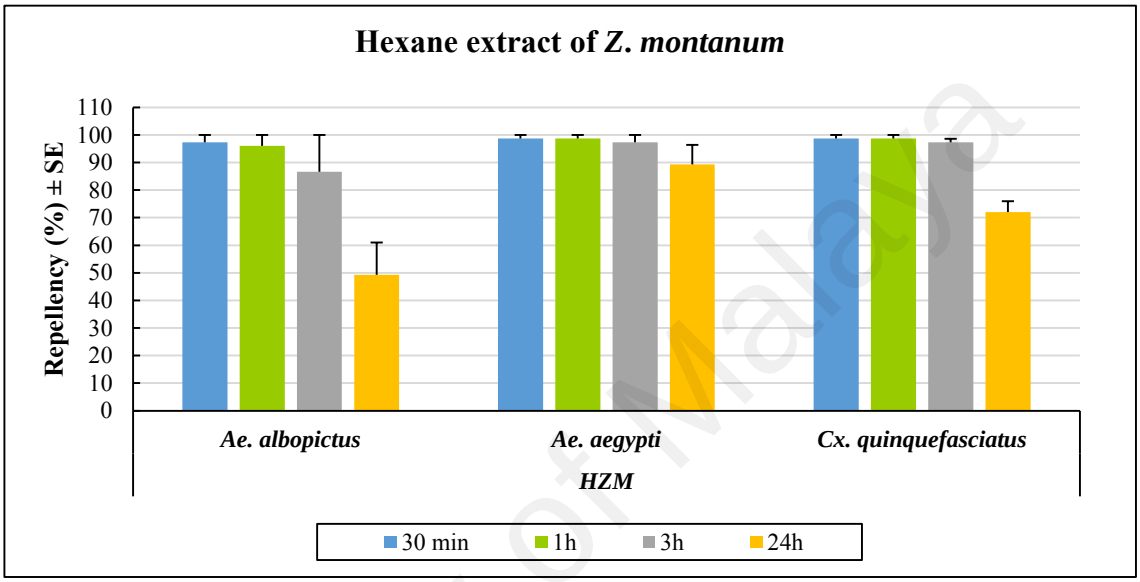


Figure 4.27: Repellency of the hexane extract of *Z. montanum* (HZM) against the three adult mosquito species at different exposure time

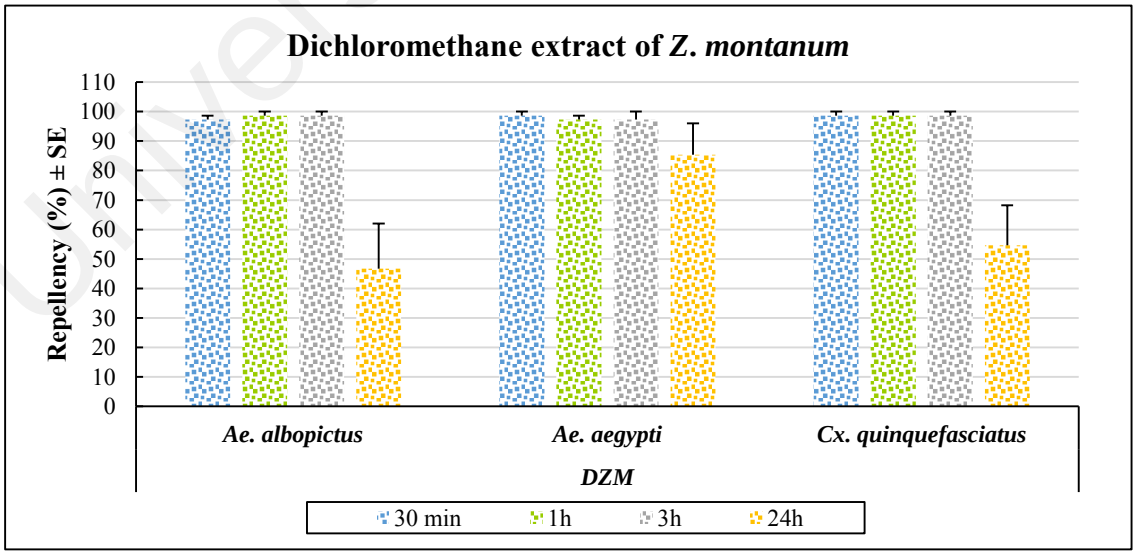


Figure 4.28: Repellency of the dichloromethane extract of *Z. montanum* (DZM) against the three adult mosquito species at different exposure time

There were significant differences in repellency obtained during the four different exposure time (30 min, 1 h, 3 h and 24 h) for each of the hexane and dichloromethane extracts of *Z. montanum* against the three mosquito species (F_{HZM}= 14.28, df= 3, $p<0.05$; F_{DZM}= 18.32, df= 3, $p<0.05$).

d) Hexane extract of *Z. spectabile*

The repellencies of the hexane extract of *Z. spectabile* (HZS) against the three mosquito species are shown in Figure 4.29. At 30 min, 1 h and 3 h exposure time, *Ae. aegypti* (100.00%) and *Cx. quinquefasciatus* (98.67%) showed high repellency as compared to *Ae. albopictus* which showed 94.67%%, 97.33%, 80.00% repellency. Then after 24 h exposure, both *Ae. aegypti* and *Cx. quinquefasciatus* still showed relatively high repellency (77.33% and 72.00%, respectively) whereas *Ae. albopictus* (28.00%) showed low repellency at the same exposure time.

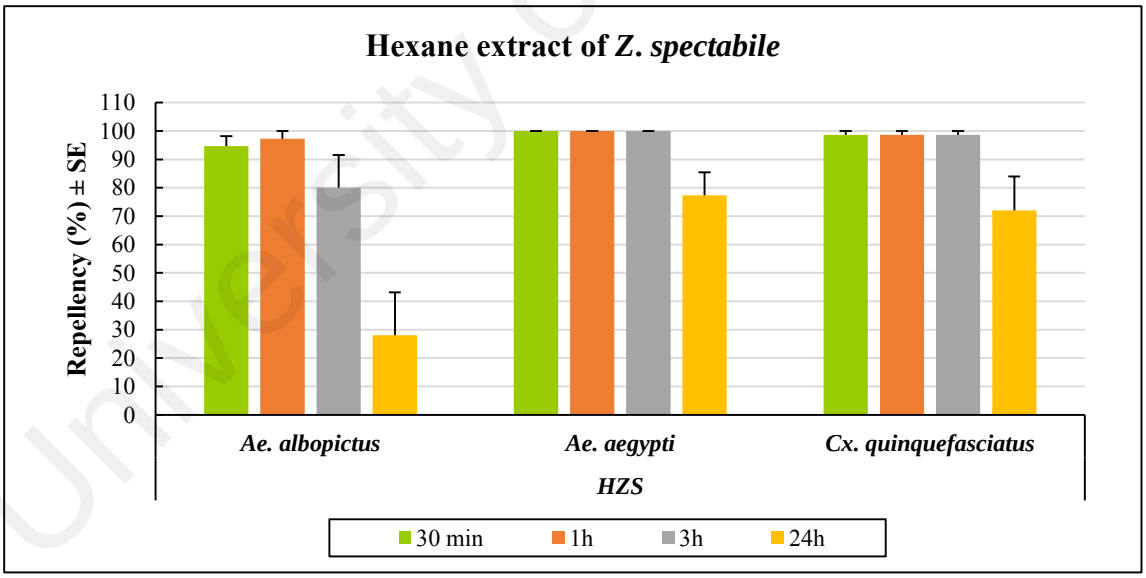


Figure 4.29: Repellency of the hexane extract of *Z. spectabile* (HZS) against the three adult mosquito species at different exposure time

e) Dichloromethane extract of *Z. spectabile*

Figure 4.30 showed the repellency effect of dichloromethane extract of *Z. spectabile* (DZS) towards the three mosquito species. All three mosquitoes showed high repellency within the range of 92.00% to 98.67%. As in the case of HZS, after 24 h exposure, both

Ae. aegypti and *Cx. quinquefasciatus* showed relatively high repellency (74.67% and 80.00%, respectively) compared to *Ae. albopictus* (24.00%) in DZS. The percentage repellency of the three mosquitoes differed significantly among the extracts and exposure time (F_{HZS}= 20.07, df= 3, $p<0.05$; F_{DZS}= 16.76, df= 3, $p<0.05$).

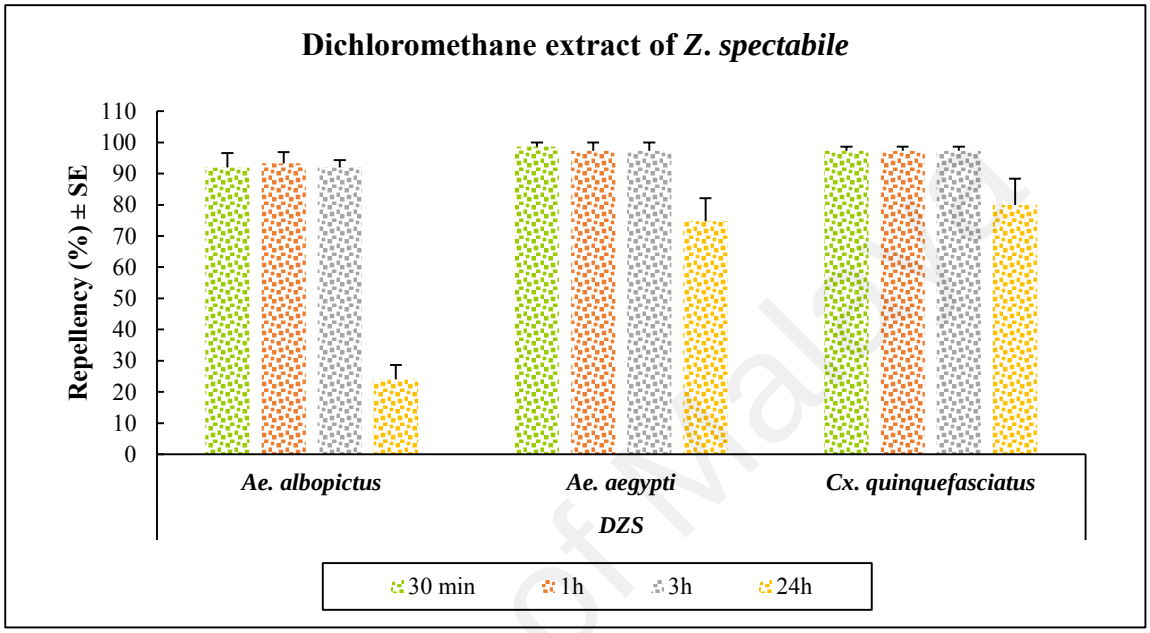


Figure 4.30: Repellency of the dichloromethane extract of *Z. spectabile* (DZS) against the three adult mosquito species at different exposure time

f) Hexane extract of *Z. zerumbet*

Figure 4.31 showed the repellencies of the three mosquito species when tested with the hexane extract of *Z. zerumbet* (HZZ). Similarly as in the case of the dichloromethane extract of *Z. spectabile* (DZS), *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* showed high repellency within the range of 97.33% to 98.67% after being tested with HZZ for 30 minutes, 1 h and 3 h exposure. At the end of 24 h exposure, *Ae. albopictus* was less repelled (30.67%) compared to *Ae. aegypti* (64.00%) and *Cx. quinquefasciatus* (72.00%).

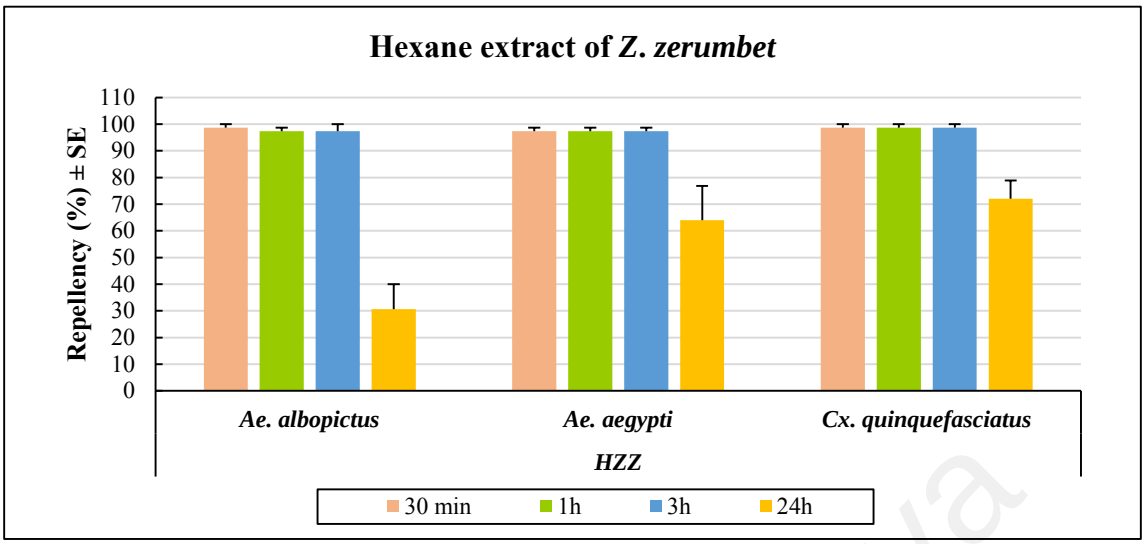


Figure 4.31: Repellency of the hexane extract of *Z. zerumbet* (HZZ) against the three adult mosquito species at different exposure time

g) Dichloromethane extract of *Z. zerumbet*

High repellency of *Ae. aegypti* (96.00% - 98.67%) and *Cx. quinquefasciatus* (93.33% - 96.00%) were reported in DZZ after being exposed for 30 minutes, 1 h and 3 h, while relatively high repellency of *Ae. albopictus* was observed after 30 min (93.33%), 1 h (86.67%) and 3 h (82.67%) after being exposed with DZZ. However, after 24 h exposure, low to relatively low repellency of the three mosquito species were detected that is *Ae. albopictus* (20.00%), *Ae. aegypti* (28.00%) and *Cx. quinquefasciatus* (40.00%) (Figure 4.32).

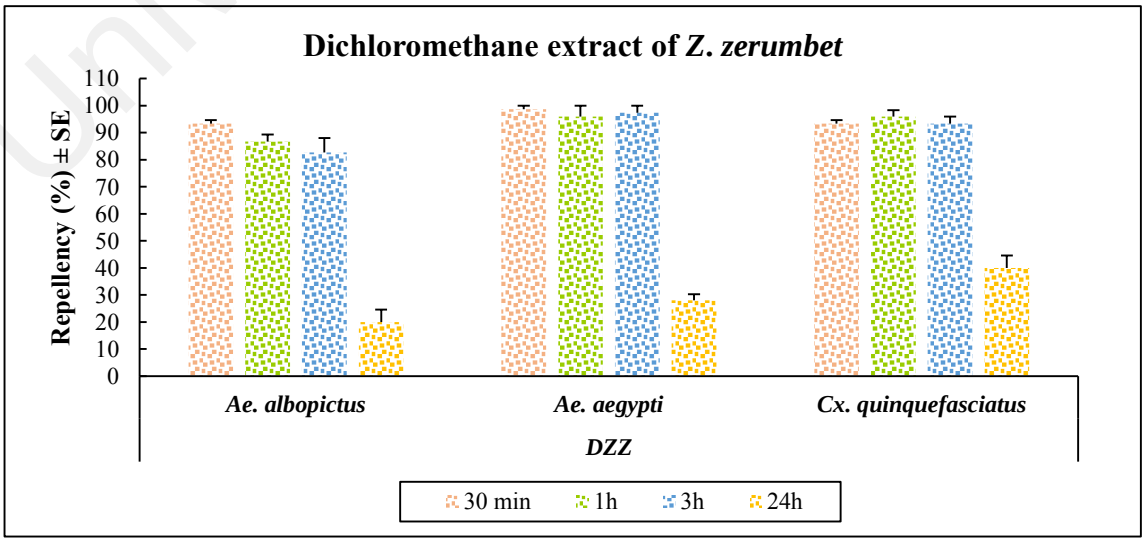


Figure 4.32: Repellency of the dichloromethane extract of *Z. zerumbet* (DZZ) against the three adult mosquito species at different exposure time

There were significant differences in repellencies among mosquito species between the exposure time and the hexane and dichloromethane extracts of *Z. zerumbet* (F_{HZZ}= 21.88, df= 3, $p<0.05$; F_{DZZ}= 20.70, df= 3, $p<0.05$). Thus, between HZZ and DZZ, relatively higher repellency of the three mosquito species was reported in HZZ than DZZ after 24 h exposure.

h) Hexane extract of *C. aeruginosa*

Results on the repellency of the hexane extract of *C. aeruginosa* (HCA) at different exposure time is presented in Figure 4.33. The result reveal that *Ae. albopictus* (93.33% - 94.67%), *Ae. aegypti* (98.67% - 100.00%) and *Cx. quinquefasciatus* (96.00% - 100.00%) were highly repelled to the extract after being exposed for 30 minutes, 1 h and 3 h. After 24 h exposure, the highest repellency was reported against *Ae. aegypti* (52.00%), followed by *Ae. albopictus* (46.67%) and *Cx. quinquefasciatus* (34.67%).

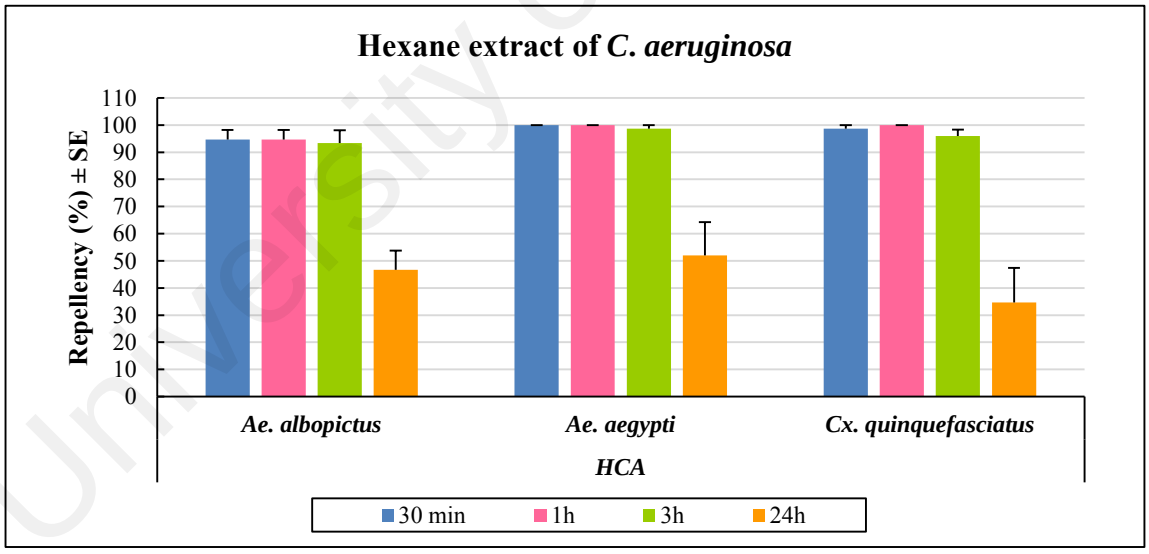


Figure 4.33: Repellency of the hexane extract of *C. aeruginosa* (HCA) against the three adult mosquito species at different exposure time

i) Dichloromethane extract of *C. aeruginosa*

Figure 4.34 shows the repellency of the dichloromethane extract of *C. aeruginosa* (DCA) against the three mosquito species at different exposure time. The result shows similar trend as in HCA, that is, after 30 minutes, 1 h and 3 h exposure, high repellency

activity within the range of 90.67% to 98.67% against *Ae. albopictus* (97.33% - 98.67%), *Ae. aegypti* (90.67% - 92.00%), and *Cx. quinquefasciatus* (94.67% - 96.00%). However, after being exposed for 24 h, both *Ae. albopictus* and *Ae. aegypti* showed moderate repellency activity (50.67% and 49.33%), but lower repellency was observed in *Cx. quinquefasciatus* (45.33%).

The different exposure time significantly increased the repellencies of the hexane and dichloromethane extracts against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* (F_{HCA}= 23.07, df= 3, $p<0.05$; F_{DCA}= 20.67, df= 3, $p<0.05$).

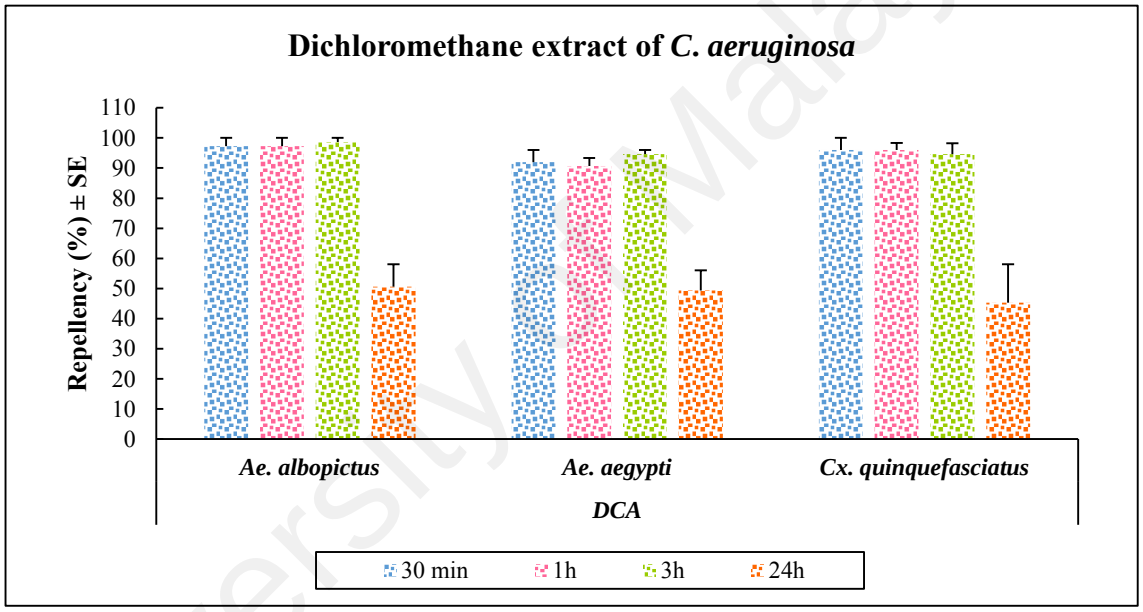


Figure 4.34: Repellency of the dichloromethane extract of *C. aeruginosa* (DCA) against the three adult mosquito species at different exposure time

The order or potency of the hexane extracts of five Zingiberaceae species in repelling *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* after 24 h exposure are as follows:

Ae. *albopictus* : HZM (49.33%) > HCA (46.67%) > HZOR (37.33%) > HZZ (30.67%) > HZS (28.00%)

Ae. *aegypti* : HZM (89.33%) > HZS (77.33%) > HZZ (64.00%) > HZOR (62.67%) > HCA (52.00%)

Cx. *quinquefasciatus* : HZM (72.00%) ≥ HZS (72.00%) ≥ HZZ (72.00%) > HZOR (65.33%) > HCA (34.67%)

Meanwhile, the order or potency of the dichloromethane extracts of five Zingiberaceae species in repelling *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* after 24 h exposure are as follows:

Ae. *albopictus* : DCA (50.67%) > DZOR (46.67%) ≥ DZM (46.67%) > DZS (24.00%) > DZZ (20.00%)

Ae. *aegypti* : DZM (85.33%) > DZS (74.67%) > DCA (49.33%) > DZOR (46.67%) > DZZ (28.00%)

Cx. *quinquefasciatus* : DZS (80.00%) > DZM (54.67%) > DZOR (53.33%) > DCA (45.33%) > DZZ (40.00%)

In conclusion, the hexane extract of *Z*. *montanum* (HZM) appeared to be the most effective extract in repelling *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* whereas the dichloromethane extract of *Z*. *zerumbet* (DZZ) was the most ineffective extract in repelling these mosquito species.

The lethal time (LT) of the hexane and dichloromethane extracts of *Z. officinale* var. *rubrum* (HZOR and DZOR), *Z. montanum* (HZM and DZM), *Z. spectabile* (HZS and DZS), *Z. zerumbet* (HZZ and DZZ) and *C. aeruginosa* (HCA and DCA) against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* were calculated after 24 h exposure and are listed in Table 4.7.

Table 4.7: The lethal time (LT_{50} and LT_{90}) values of five Zingiberaceae species against adults of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*

Zingiberaceae species	Solvents	Mosquito species	*LT_{50} (h)	LT_{90} (h)
Z. officinale* var. *rubrum	Hexane	*Ae. albopictus*	3.86	2.50
		Ae. aegypti	6.24	2.59
		Cx. quinquefasciatus	4.93	3.13
	Dichloromethane	*Ae. albopictus*	4.07	3.08
		Ae. aegypti	5.00	1.15
		Cx. quinquefasciatus	5.17	2.48
Z. montanum	Hexane	*Ae. albopictus*	4.20	2.36
		Ae. aegypti	21.50	4.63
		Cx. quinquefasciatus	6.53	3.07
	Dichloromethane	*Ae. albopictus*	4.69	2.52
		Ae. aegypti	14.82	3.78
		Cx. quinquefasciatus	3.56	2.62
Z. spectabile	Hexane	*Ae. albopictus*	3.73	1.90
		Ae. aegypti	4.95	3.55
		Cx. quinquefasciatus	6.53	2.72
	Dichloromethane	*Ae. albopictus*	3.78	2.02
		Ae. aegypti	7.53	3.02
		Cx. quinquefasciatus	13.74	3.24
Z. zerumbet	Hexane	*Ae. albopictus*	3.88	2.57
		Ae. aegypti	10.12	0.46
		Cx. quinquefasciatus	6.53	3.07
	Dichloromethane	*Ae. albopictus*	3.48	1.61
		Ae. aegypti	3.82	2.50
		Cx. quinquefasciatus	4.80	1.91
C. aeruginosa	Hexane	*Ae. albopictus*	5.14	2.00
		Ae. aegypti	4.40	2.87
		Cx. quinquefasciatus	3.93	2.70
	Dichloromethane	*Ae. albopictus*	5.00	2.49
		Ae. aegypti	6.82	2.10
		Cx. quinquefasciatus	4.83	2.18

*LT= Lethal Time

Based on the lethal time values, the shortest time for *Ae*. *albopictus* to enter the bait was 3.48 h and recorded in DZZ; for *Ae*. *aegypti* was also recorded in DZZ at 3.82 h; and for *Cx*. *quinquefasciatus*, was recorded in DZM at 3.56 h. Meanwhile, the longest time to prevent 50% of *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* to enter the bait was 5.14 h and recorded in HCA; 21.50 h in HZM and 13.74 h in DZS, respectively.

4.6.2 The blood-feeding inhibition of the hexane and dichloromethane extracts of five Zingiberaceae species against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*

a) Hexane extract of *Z. officinale* var. *rubrum*

Figure 4.35 showed the blood-feeding inhibition of the hexane extract of *Z*. *officinale* var. *rubrum* (HZOR) against the three mosquito species at 30 minutes, 1h, 3h and 24h exposure. The results revealed that *Ae*. *aegypti* showed blood-feeding inhibition at all 3 exposure time (30 min, 1 h and 3 h) but at very low percentage (2.67%). Blood-feeding inhibition against *Ae*. *albopictus* was observed after 1 h and 3 h exposure (6.67% and 9.33%, respectively) while *Cx*. *quinquefasciatus* did not show any blood-feeding inhibition except at 24 h exposure. After 24 h exposure, *Ae*. *aegypti* (33.33%) and *Cx*. *quinquefasciatus* (29.33%) showed lower blood-feeding inhibition than *Ae*. *albopictus* which showed moderate blood-feeding inhibition (50.67%).

There was significant differences on the blood-feeding inhibition of the three mosquito species in HZOR at different exposure time with $F= 22.45$, $df= 3$, $p<0.05$.

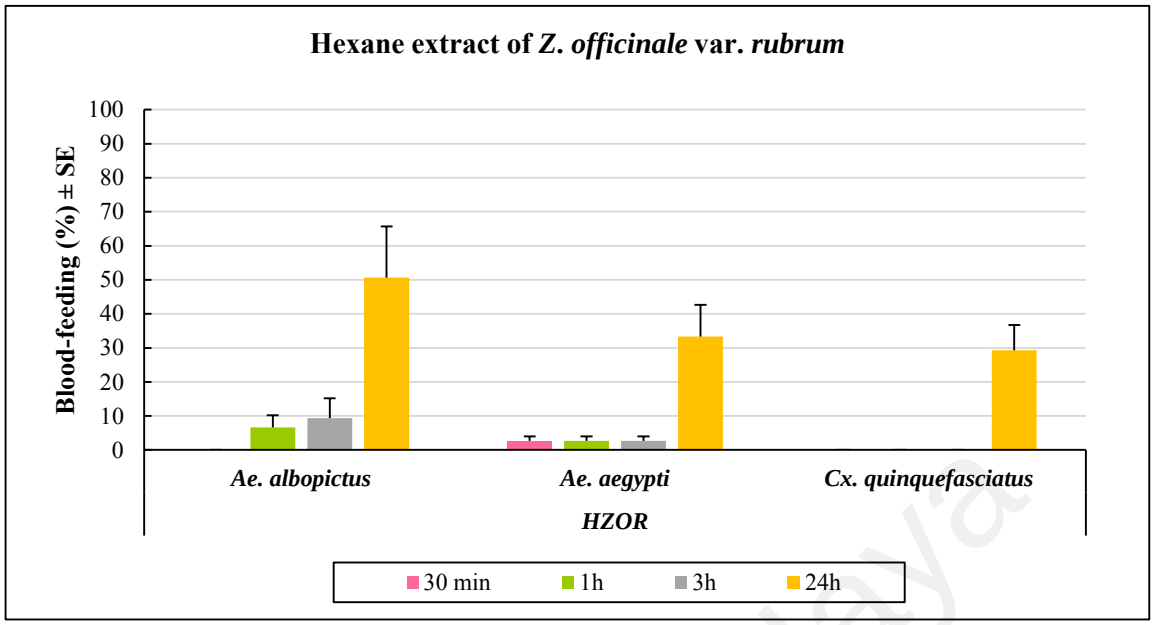


Figure 4.35: Blood-feeding inhibition of the three mosquito species in the hexane extract of *Z. officinale* var. *rubrum* (HZOR) at different exposure time

b) Dichloromethane extract of *Z. officinale* var. *rubrum*

The dichloromethane extract of *Z. officinale* var. *rubrum* (DZOR) induced blood-feeding inhibition against *Ae. aegypti* and *Cx. quinquefasciatus* at all exposure time while blood-feeding inhibition against *Ae. albopictus* was recorded only after 24 h exposure. Throughout the three exposure time (30 min, 1 h and 3 h), blood-feeding inhibition of the mosquito was higher against *Ae. aegypti* (12.00%, 16.00% and 22.67%, respectively) than *Cx. quinquefasciatus* (1.33%). However, after 24 h exposure, moderate blood-feeding inhibition was showed against both *Ae. aegypti* (53.33%) and *Cx. quinquefasciatus* (44.00%) while *Ae. albopictus* showed relatively low blood-feeding activity (30.67%) (Figure 4.36).

There was a significant difference in blood-feeding inhibition for the dichloromethane extract of *Z. officinale* var. *rubrum* (DZOR) against the three mosquito species at different exposure time with $F= 19.68$, $df= 3$, $p<0.05$. Thus, HZOR induced more blood-feeding inhibition against *Ae. albopictus* than others whereas DZOR induced more blood-feeding inhibition against *Ae. aegypti* than *Ae. albopictus* and *Cx. quinquefasciatus*.

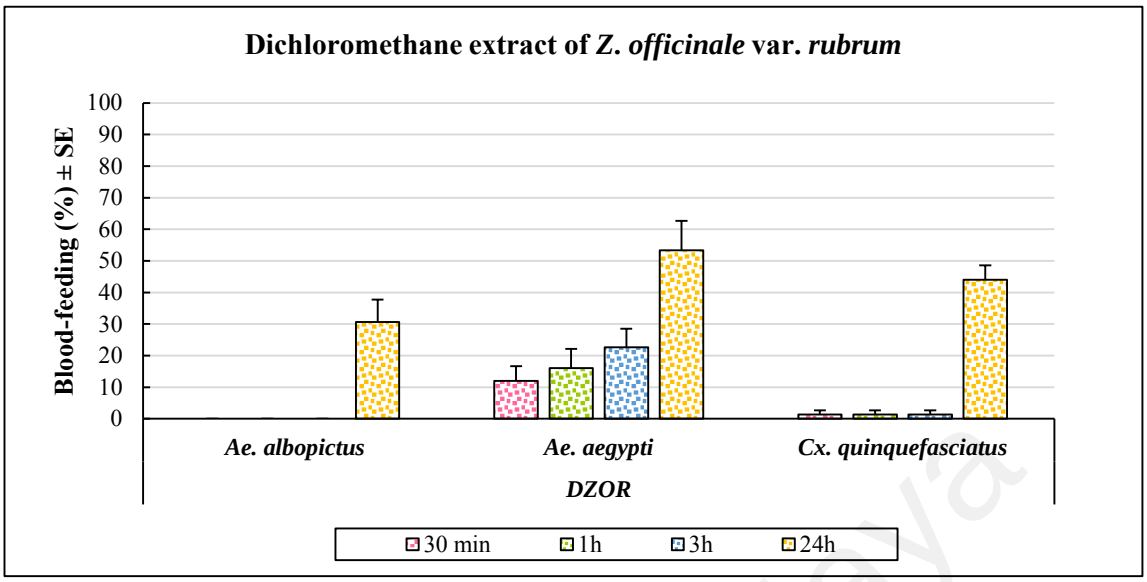


Figure 4.36: Blood-feeding inhibition of the three mosquito species in the dichloromethane extract of *Z. officinale* var. *rubrum* (DZOR) at different exposure time

c) Hexane extract of *Z. montanum*

The blood-feeding inhibition of the hexane extract of *Z. montanum* (HZM) against the three mosquito species at 30 minutes, 1 h, 3 h and 24 h exposure are illustrated in Figure 4.37. The results show that HZM induced blood-feeding activity against all three mosquito species at all exposure time. After 30 minutes to 3 h exposure, HZM induced more blood-feeding inhibition against *Ae. albopictus* (2.67% - 12.00%) than *Ae. aegypti* (1.33%) and *Cx. quinquefasciatus* (1.33% - 2.67%). Blood-feeding activity against *Ae. albopictus* (37.33%) was higher than *Ae. aegypti* (10.67%) and *Cx. quinquefasciatus* (22.67%) after 24 h exposure.

d) Dichloromethane extract of *Z. montanum*

Figure 4.38 showed similar trend with HZM where low blood-feeding inhibition of *Ae. albopictus* (1.33% - 2.67%), *Ae. aegypti* (1.33% - 2.67%) and *Cx. quinquefasciatus* (1.33% - 2.67%) were observed after being tested with the dichloromethane extract of *Z. montanum* (DZM) at 30 minutes, 1 h and 3 h exposure. After being exposed for 24 h,

DZM induced higher blood-feeding activity against *Ae. albopictus* (41.33%) than *Cx. quinquefasciatus* (34.67%) and *Ae. aegypti* (14.67%). Therefore, both HZM and DZM induced more blood-feeding activity against *Ae. albopictus* than *Ae. aegypti* and *Cx. quinquefasciatus*.

There were significant differences in blood-feeding inhibition of the three mosquito species at different exposure time in both HZM and DZM (F_{HZM}= 14.05, df= 3, $p<0.05$; F_{DZM}= 18.10, df= 3, $p<0.05$).

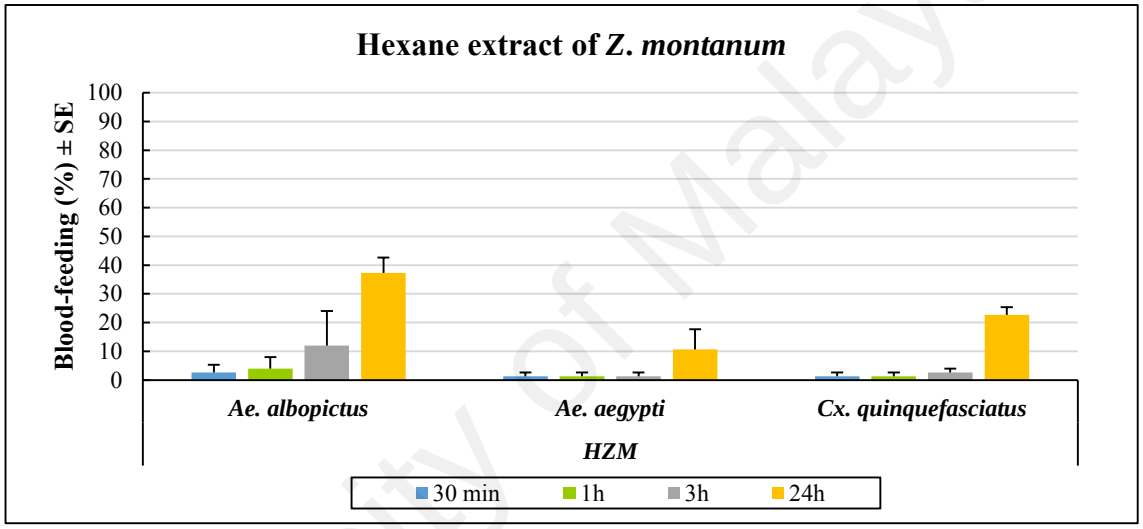


Figure 4.37: Blood-feeding inhibition of the three mosquito species in the hexane extract of *Z. montanum* (HZM) at different exposure time

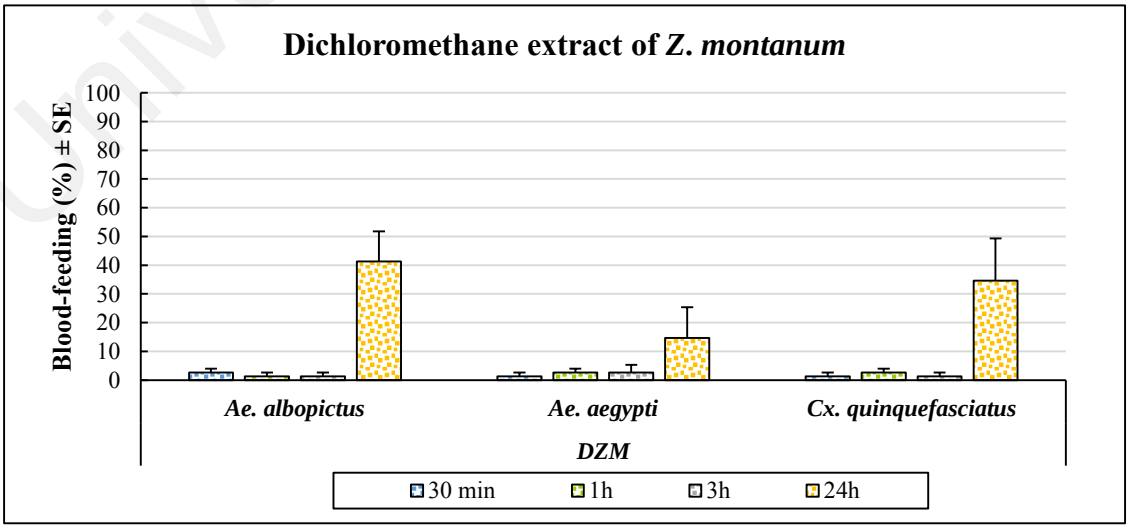


Figure 4.38: Blood-feeding inhibition of the three mosquito species in the dichloromethane extract of *Z. montanum* (DZM) at different exposure time

e) Hexane extract of *Z. spectabile*

The blood-feeding inhibition of the hexane extract of *Z. spectabile* (HZS) against the three mosquito species was shown in Figure 4.39. Blood-feeding activity against *Ae. albopictus* and *Cx. quinquefasciatus* were observed at all exposure time (30 min, 1 h, 3 h and 24 h) while blood-feeding activity against *Ae. aegypti* was observed only after 24 h exposure. Low to very low blood-feeding inhibition was reported in both *Ae. albopictus* (2.67% to 20.00%) and *Cx. quinquefasciatus* (1.33%) after 30 min, 1 h and 3 h exposure. However, after 24 h exposure, *Ae. albopictus* showed moderate blood-feeding activity (42.67%) while *Cx. quinquefasciatus* and *Ae. aegypti* showed relatively low blood-feeding activity (22.67% and 28.00%, respectively).

f) Dichloromethane extract of *Z. spectabile*

Blood-feeding activity of all three mosquito species was recorded at all exposure time after being tested with the dichloromethane extract of *Z. spectabile* (DZS). After 30 min, 1 h and 3 h exposure, very low blood-feeding activity was reported against the three mosquito species that is *Ae. albopictus* (4.00% to 8.00%), *Ae. aegypti* (1.33% to 2.67%) and *Cx. quinquefasciatus* (2.67%). However, after 24 h exposure, *Ae. aegypti* (22.67%) and *Cx. quinquefasciatus* (17.33%) showed low blood-feeding activity compared to relatively high in *Ae. albopictus* (69.33%) (Figure 4.40). Thus, HZS was better in inhibiting the blood-feeding activity of *Ae. albopictus* than *Ae. aegypti* and *Cx. quinquefasciatus*.

The blood-feeding inhibition of the three mosquito species were significantly difference at different exposure time in HZS ($F= 19.76$, $df= 3$, $p<0.05$) and DZS ($F= 17.21$, $df= 3$, $p<0.05$)

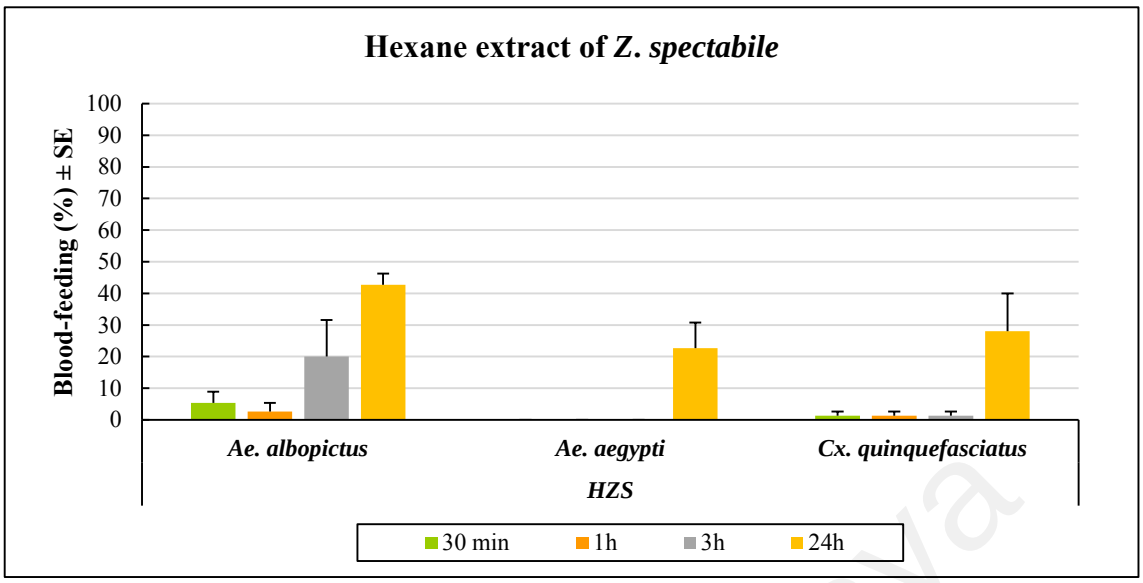


Figure 4.39: Blood-feeding inhibition of the three mosquito species in the hexane extract of *Z. spectabile* (HZS) at different exposure time

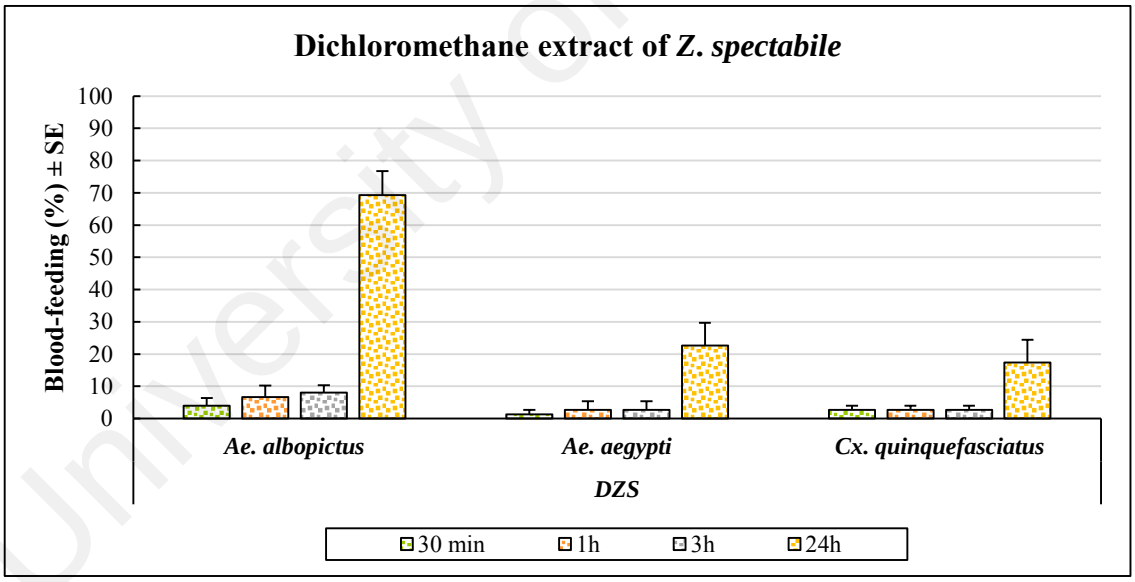


Figure 4.40: Blood-feeding inhibition of the three mosquito species in the dichloromethane extract of *Z. spectabile* (DZS) at different exposure time

g) Hexane extract of *Z. zerumbet*

In the hexane extract of *Z. zerumbet* (HZZ), very low blood-feeding inhibition (1.33% - 2.67%) of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* were recorded at the first 3 h exposure (30 min, 1 h and 3 h). However, after 24 h exposure, relatively low blood-feeding inhibition was recorded against *Ae. aegypti* (32.00%) and *Cx. quinquefasciatus* (28.00%) compared to *Ae. albopictus* which showed slightly moderate blood-feeding inhibition (45.33%) (Figure 4.41). The blood-feeding inhibition of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* were significantly different among the exposure time ($F= 21.88$, $df= 3$, $p<0.05$).

h) Dichloromethane extract of *Z. zerumbet*

Figure 4.42 shows the blood-feeding inhibition for the dichloromethane extract of *Z. zerumbet* (DZZ) against the three mosquito species. There were significant differences in blood-feeding inhibition among the mosquito species at different exposure time ($F= 20.55$, $df= 3$, $p<0.05$). At the first 3 h exposure (30 min, 1 h and 3 h), the blood-feeding inhibition of *Ae. aegypti* (1.33% to 4.00%) and *Cx. quinquefasciatus* (4.00% to 6.67%) were lower than *Ae. albopictus* (6.67% to 17.33%). All the three mosquito species showed moderate blood-feeding inhibition after being exposed for 24 h but differ slightly among the species (*Ae. albopictus*= 50.67%; *Ae. aegypti*= 52.00%, and *Cx. quinquefasciatus*= 53.33%).

The results of blood-feeding inhibition of *Ae. albopictus, Ae. aegypti and Cx. quinquefasciatus* mosquito after being exposed to the hexane extract of *Z. zerumbet* showed almost similar results with those from the dichloromethane extract. However, the dichloromethane extract of *Z. zerumbet* induced more blood-feeding inhibition against the three mosquitoes compared to the hexane extract.

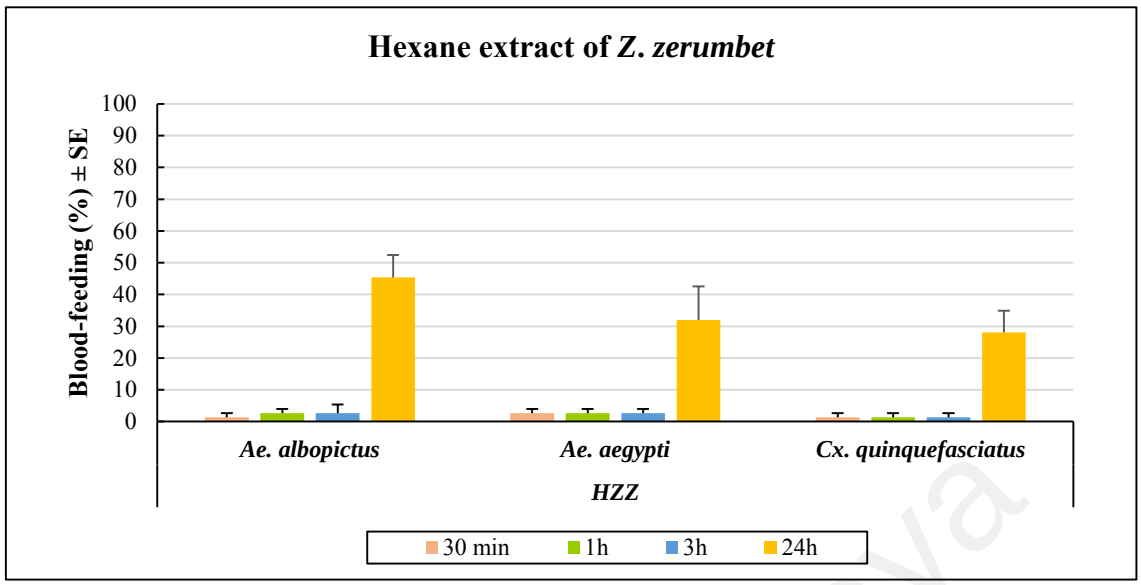


Figure 4.41: Blood-feeding inhibition of the three mosquito species in the hexane extract of *Z. zerumbet* (HZZ) at different exposure time

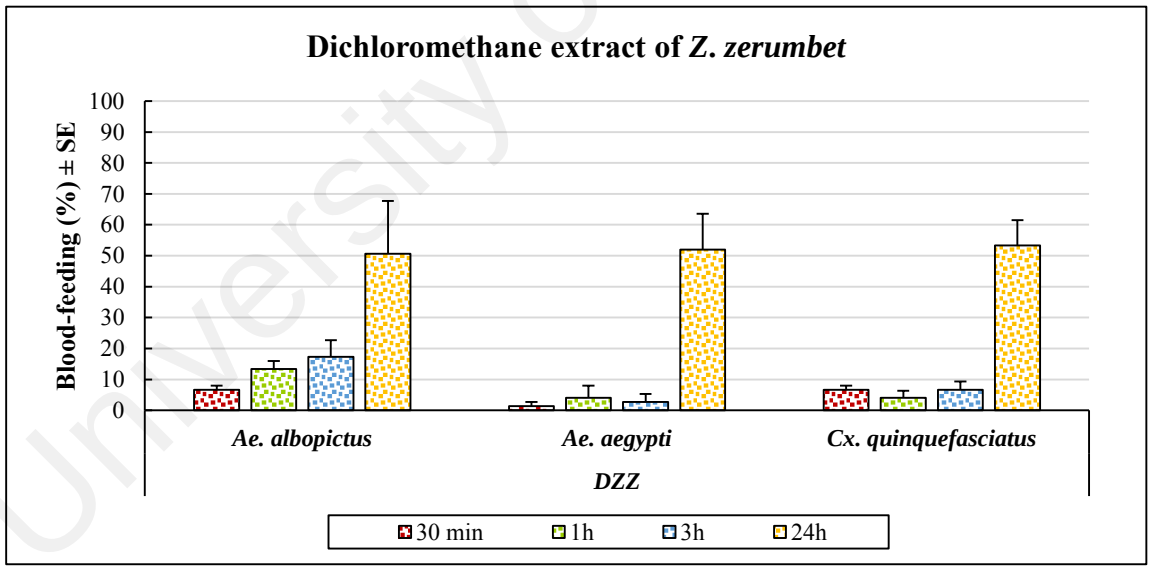


Figure 4.42: Blood-feeding inhibition of the three mosquito species in the dichloromethane extract of *Z. zerumbet* (DZZ) at different exposure time

i) Hexane extract of *C. aeruginosa*

The blood-feeding inhibition of the hexane extract of *C. aeruginosa* (HCA) against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* is presented in Figure 4.43. The results show that all three mosquito species exhibit blood-feeding activity for all exposure time. At 30 min, 1 h and 3 h exposure, low blood-feeding inhibition was observed against *Ae. albopictus* (5.33% to 6.67%) but higher than *Ae. aegypti* (0.00% to 1.33%) and *Cx. quinquefasciatus* (0.00% to 4.00%). Interestingly, after 24 h exposure, *Ae. aegypti* (42.67%) and *Cx. quinquefasciatus* (48.00%) showed moderate blood-feeding inhibition but still higher than *Ae. albopictus* (29.33%).

j) Dichloromethane extract of *C. aeruginosa*

In the dichloromethane extract of *C. aeruginosa* (DCA), all three mosquitoes showed low blood-feeding activity at 30 minutes, 1 h and 3 h exposure, but *Ae. aegypti* showed the highest (5.33% to 9.33%) followed by *Cx. quinquefasciatus* (4.00% to 5.33%) and *Ae. albopictus* (1.33% to 2.67%). Similarly to HCA, slightly lower blood-feeding activity against *Ae. albopictus* (36.00%) was recorded than *Ae. aegypti* (37.33%) and *Cx. quinquefasciatus* (48.00%) after being exposed for 24 h in DCA (Figure 4.44). Therefore, both HCA and DCA induced more blood-feeding activity against *Cx. quinquefasciatus* than *Aedes* species.

There were significant differences in the blood-feeding inhibition of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* among the exposure time in HCA ($F= 23.07$, $df= 3$, $p<0.05$) and DCA ($F= 20.63$, $df= 3$, $p<0.05$).

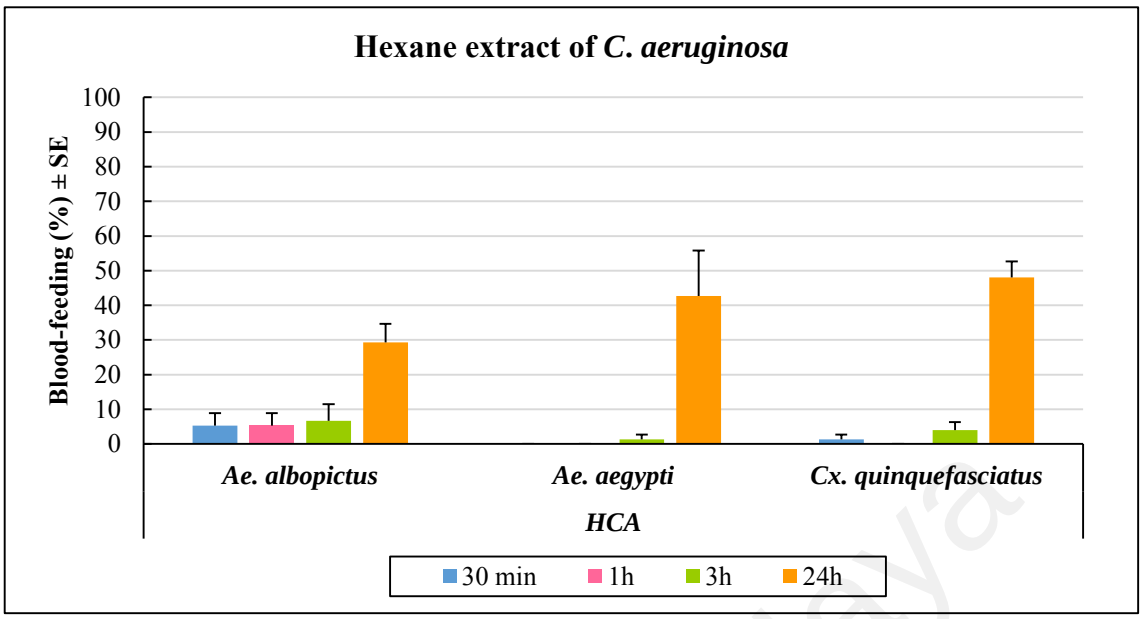


Figure 4.43: Blood-feeding inhibition of the three mosquito species in the hexane extract of *C. aeruginosa* (HCA) at different exposure time

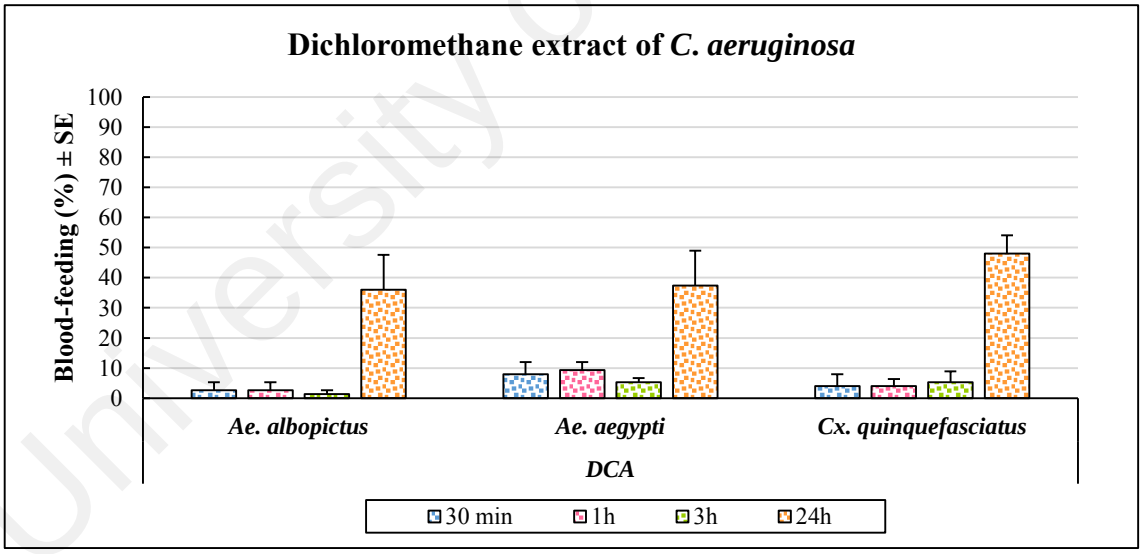


Figure 4.44: Blood-feeding inhibition of the three mosquito species in the dichloromethane extract of *C. aeruginosa* (DCA) at different exposure time

The order or potency of the hexane extracts of five Zingiberaceae species for blood-feeding activity against the three mosquitoes are listed as follows:

Ae. albopictus : HCA (29.33%) < HZM (37.33%) < HZS (42.67%) < HZZ (45.33%) < HZOR (50.67%)

Ae. aegypti : HZM (10.67%) < HZS (22.67%) < HZZ (32.00%) < HZOR (33.33%) < HCA (42.67%)

Cx. quinquefasciatus : HZM (22.67%) < HZS (28.00%) ≤ HZZ (28.00%) < HZOR (29.33%) < HCA (48.00%)

The order or potency of the dichloromethane extracts of five Zingiberaceae species for blood-feeding activity against the three mosquitoes are listed as follows:

Ae. albopictus : DZOR (30.67%) < DCA (36.00%) < DZM (41.33%) < DZZ (50.67%) < DZS (69.33%)

Ae. aegypti : DZM (14.67%) < DZS (22.67%) < DCA (37.33%) < DZZ (52.00%) <DZOR (53.33%)

Cx. quinquefasciatus : DZS (17.33%) < DZM (34.67%) < DZOR (44.00%) < DCA (48.00%) < DZZ (53.33%)

Thus, among the hexane and dichloromethane extracts of five Zingiberaceae species, the blood-feeding activity of *Ae. albopictus* were highly induced by HCA and DZOR while for *Ae. aegypti* were by HZM and DZM. HZM and DZS exhibited more blood-feeding activity against *Cx. quinquefasciatus* than the other extracts.

4.6.3 Effect of the hexane and dichloromethane extracts of five Zingiberaceae species on mortality activity of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*

After being tested with the hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* for 30 minutes, 1 h and 3 h exposure, none of the mosquito species died. However, after 24 h exposure, the mortality of the mosquitoes were observed and is given in Table 4.8.

As can be seen in Table 4.8, the hexane and dichloromethane extracts of five Zingiberaceae species induced higher mortality towards *Ae. albopictus* than *Ae. aegypti* and *Cx. quinquefasciatus*. Five extracts, that is, hexane extract of *Z. spectabile* (HZS), hexane extract of *Z. zerumbet* (HZZ), hexane extract of *C. aeruginosa* (HCA), dichloromethane extract of *Z. officinale* var. *rubrum* (DZOR) and dichloromethane extract of *Z. zerumbet* (DZZ) were relatively effective in killing *Ae. albopictus* ranging from 22.67% to 29.33%. Dichloromethane extract of *Z. zerumbet* (DZZ) was slightly effective against *Ae. aegypti* (20.00%) whereas hexane extract of *C. aeruginosa* (HCA) was moderately effective against *Cx. quinquefasciatus* (17.33%).

Hexane and dichloromethane extracts of *Z. montanum* (HZM, DZM) and dichloromethane extract of *Z. officinale* var. *rubrum* (DZOR) did not kill *Ae. aegypti* but killed *Ae. albopictus* (13.33%, 12.00% and 22.67%, respectively) and *Cx. quinquefasciatus* (5.33%, 10.67% and 2.67%, respectively). Hexane extract of *Z. spectabile* (HZS) moderately killed *Ae. albopictus* (29.33%) but did not kill both *Ae. aegypti* and *Cx. quinquefasciatus*. On the other hand, hexane extract of *Z. zerumbet* (HZZ) killed both *Ae. albopictus* (24.00%) and *Ae. aegypti* (4.00%) at lower percentage but did not kill *Cx. quinquefasciatus*.

Table 4.8: The percentage mortality of adults *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* after 24 hours exposure in five Zingiberaceae species

Solvents	Plant species	Mortality (%) ± SE		
		Ae. albopictus	***Ae. aegypti***	***Cx. quinquefasciatus***
Hexane	*Z. officinale* var. *rubrum*	12.00 ± 12.00	4.00 ± 2.31	5.33 ± 2.67
	Z. montanum	13.33 ± 13.33	0.00 ± 0.00	5.33 ± 3.53
	Z. spectabile	29.33 ± 17.33	0.00 ± 0.00	0.00 ± 0.00
	Z. zerumbet	24.00 ± 6.11	4.00 ± 2.31	0.00 ± 0.00
	C. aeruginosa	24.00 ± 4.62	5.33 ± 1.33	17.33 ± 8.74
Dichloromethane	*Z. officinale* var. *rubrum*	22.67 ± 16.71	0.00 ± 0.00	2.67 ± 2.67
	Z. montanum	12.00 ± 12.00	0.00 ± 0.00	10.67 ± 2.67
	Z. spectabile	6.67 ± 4.81	2.67 ± 1.33	2.67 ± 1.33
	Z. zerumbet	29.33 ± 13.92	20.00 ± 10.58	6.67 ± 6.67
	C. aeruginosa	13.33 ± 5.33	13.33 ± 7.42	6.67 ± 6.67

Overall, for all extracts, mortality of *Ae*. *albopictus* ranged from 6.67% to 29.33%; for *Ae*. *aegypti* (except for dichloromethane extract of *Z*. *zerumbet* (DZZ)) ranged from 2.67% to 20.00%; and for *Cx*. *quinquefasciatus* (except for hexane extracts of *Z*. *spectabile* and *Z*. *zerumbet* (HZS and HZZ)) ranged from 2.67% to 17.33%.

There were significant differences between the mortality of *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* and the exposure time in HZOR ($F= 0.32$, $df= 2$, $p>0.05$) but not significant in DZOR ($F= 5.68$, $df= 3$, $p>0.05$). In HZM and HZS, there were no significant differences on the mortality of the three mosquitoes among the exposure time ($F_{HZM}= 6.28$, $df = 3$, $p>0.05$; $F_{HZS}= 6.17$, $df= 3$, $p>0.05$) but in DZM and DZS, the mortality of the mosquitoes were recorded as significantly different among the exposure time ($F_{DZM}= 13.07$, $df=3$, $p<0.05$; $F_{DZS}= 20.90$, $df= 3$, $p<0.05$). The exposure time significantly affected the mortality of *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* in HZZ ($F= 16.82$, $df= 3$, $p<0.05$) and HCA ($F= 29.40$, $df=3$, $p<0.05$) as well as in DZZ and DCA ($F= 24.97$, $df=3$, $p<0.05$).

In summary, the hexane and dichloromethane extracts of *Z*. *officinale* var. *rubrum* (HZOR and DZOR), *Z*. *montanum* (HZM and DZM), *Z*. *spectabile* (HZS and DZS), *Z*. *zerumbet* (HZZ and DZZ) and *C*. *aeruginosa* (HCA and DCA) were relatively effective for the repellency activity, blood-feeding inhibition and mortality activity against *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus*. Most of the results for *Ae*. *aegypti* and *Cx*. *quinquefasciatus* relatively similar.

Among the hexane extracts, HZM can be considered as the most potent extract in repelling *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* (49.33%, 89.33% and 72.00%, respectively) whereas among the dichloromethane extracts, DCA was the most effective to repel *Ae*. *albopictus* (50.64%), DZM for *Ae*. *aegypti* (85.33%) and DZS for *Cx*. *quinquefasciatus* (80.00%). The highest blood-feeding inhibition against *Ae*.

albopictus was reported in HZOR (50.67%) and DZS (69.33%); against *Ae*. *aegypti* was in HCA (42.67%) and DZOR (53.33%); and against *Cx*. *quinquefasciatus* was in HCA (48.00%) and DZZ (53.33%). The highest mortality of *Ae*. *albopictus* was in HZS (29.33%) whereas the highest mortality for *Ae*. *aegypti* and *Cx*. *quinquefasciatus* was in HCA (5.33% and 17.33%, respectively). On the other hand, DZZ induced high mortality of both *Ae*. *albopictus* (29.33%) and *Ae*. *aegypti* (20.00%) while DZM induced high mortality of *Cx*. *quinquefasciatus* (10.67%).

CHAPTER 5: DISCUSSION

5.1 THE PERCENTAGE YIELD AND CHEMICAL COMPOSITIONS OF THE HEXANE AND DICHLOROMETHANE EXTRACTS OF FIVE ZINGIBERACEAE SPECIES

The total percentage yield of the hexane extracts of *Z. officinale* var. *rubrum, Z. montanum, Z. spectabile, Z. zerumbet* and *C. aeruginosa* varies from 1.72 – 4.33%, whereas the total percentage yield obtained in the dichloromethane extracts varies from 1.37 – 5.90%. The total percentage yield of the dichloromethane extracts was slightly higher than in the hexane extracts. Several factors can influence the differences in the percentage yields, such as: the duration of extraction. According to Mejdoub and Katsiotis (1998), the longer the duration of the extraction time, the higher the yield will be obtained. Since the time required in extraction process using hexane solvent in this study was shorter than using dichloromethane solvent, therefore, the yield obtained in the hexane extracts was lower than in the dichloromethane extracts.

The other factor which can affect the different percentage yield is the polarities of the solvents. The nonpolar to moderately polar solute contain high moisture levels (King, 1990). Hexane (a non-polar solvent) can evaporate rapidly so that the extraction time required will be short. In contrast, dichloromethane which is moderately polar, need more time to evaporate. As a result, yield extracted in the hexane extracts was lower than in the dichloromethane extracts.

In addition, differences in plant species and their climatic or geographical areas, methods of extraction and plant-related factors, including parts of plant, rearing condition,

maturation of the harvested plant and plant storage or preservation, were the other factors which can influence the percentage yield (Sutthanont *et al*., 2010).

In this study, a total of 43, 82, 50, 51 and 74 compounds were identified from the hexane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* whereas a total of 43, 70, 60, 44 and 58 compounds were found in the dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa*, respectively. Among these compounds, zingerone, 1,1'-ethylenebisdecalin, zerumbone, trans-dihydrocarvone, carbendazim, terpinen-4-ol, zingiberene, β-sesquiphellandrene, camphene, α-caryophyllene, tropolone and camphor were recorded as several major compounds found in the hexane and dichloromethane extracts of the five Zingiberaceae species. These major compounds were also reported from previous studies but with only a few studies published on the chemical constituents of plant extracts of the Zingiberaceae species used in this study. Hamad *et al*. (2016) reported that the most abundant constituents of the hexane crude extract of *Zingiber officinale* are α-zingiberene, β-sesquiphellandrene, zingerone, β-farnesene and 4-(4-hydroxy- 3-methoxyphenyl)-2-butanone. The major compounds such as 4-gingerol, (6)-dehydrogingerdione and (6)-dihydrogingerdione which were isolated from the petroleum ether extract of the rhizome of *Zingiber officinale* have been reported by Rahuman *et al*. (2008). A study by Chien *et al*. (2008) isolated the rhizome of *Z. zerumbet* using ethyl acetate and found that 3-methyl kaempferol, kaempferol-3-O-(2,4-di-O-acetyl- α -l-rhamnopyranoside) and kaempferol-3-O-(3,4-di-O-acetyl- α -l-rhamnopyranoside) are the major compounds. Results from several studies revealed that Geranyl acetate, β-caryophyllene, terpinen-4-ol, β-sesquiphellandrene, sabinene, β-pinene, caryophyllene oxide, α-terpineol, zerumbone, humulenol-II, zerumbone oxide, α-humulene zedoarazol, curcumenol, camphor, isocurcumenol, curcumenol, β-elemene were also identified as the

main components from the rhizomes of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* but these compounds were isolated from the essential oils (Malek *et al*., 2005; Sivasothy *et al*., 2011; Bordoloi *et al*., 1999; Bhuiyan *et al*., 2008; Sirat *et al*., 2001; Sivasothy *et al*., 2012; Matthes *et al*., 1980; Srivastava *et al*., 2000; Sutthanont *et al*., 2010; Sukari *et al*., 2007; Sirat *et al*., 1998; Jantan *et al*., 1999; Jarikasem *et al*., 2003). Variations in the quantity and the composition of secondary plant compounds is determined by many different factors such as genetic configuration and growth stage of the plant as well as external influences like climate, weather and soil conditions (Larsen *et al*., 1999; Aschenbroich, 2015).

5.2 LARVICIDAL ACTIVITY OF THE HEXANE AND DICHLOROMETHANE EXTRACTS OF FIVE ZINGIBERACEAE SPECIES AGAINST *AE. ALBOPICTUS*, *AE. AEGYPTI* AND *CX. QUINQUEFASCIATUS*

5.2.1 Effect of the exposure time and concentrations on the percentage mortality of the larvae

The effect of exposure time and concentrations are very important for the values of percentage mortality in the tested extracts. When exposed to the higher extract concentrations and longer exposure time, more larvae showed toxic symptoms that led to an increase in mortality values. Thus, higher mortality of mosquito larvae was mostly observed at 300 mg/L and 200 mg/L than at 70 mg/L and 100 mg/L, as well as after 24 h exposure than 1 h, 3 h and 6 h exposure. As a result, very short time of exposure and low concentrations of the tested extracts resulted in moderate to low mortality rates (Benelli *et al*., 2013).

For an example, in Figure 4.1, the percentage mortality of the hexane extract of *Z. officinale* var. *rubrum* against *Ae. albopictus* larvae increased from 1 h to 24 h exposure

at all the concentrations. At 1 h exposure, no knockdown (KD) activity was detected at the lower concentrations (70 mg/L and 100 mg/L), whereas the KD activity was noticeable at the concentrations of 200 mg/L and 300 mg/L with 4.00% and 13.33%. Then after 3 h exposure, low KD activity (8.00% - 10.68%) was reported at the lower concentrations (70 mg/L and 100 mg/L) while moderate to high KD activity (46.68% - 85.33%) was recorded at 200 mg/L and 300 mg/L. After 6 h exposure, the KD activity continued to increase at all the concentrations (< 25.00% at 70 and 100 mg/L; > 80.00% at 200 and 300 mg/L). After being exposed for 24 h, the mortality of the larvae was relatively moderate at the lower concentrations (33.33% at 70 mg/L and 36.00% at 100 mg/L) and high at the higher concentrations (98.67% at 200 mg/L and 100.00% at 300 mg/L). These findings showed that concentrations and exposure time of test substances affected degree of toxicity, mortality speed and mortality rates. Murugan *et al*. (2012) and Kovendan *et al*. (2012) studied the ethanol extract of *Citrus sinensis* and leaf oil of *Morinda citrifolia* against *Anopheles stephensi*. They found that the mortality of the larvae increase with increase in concentration. Kalaivani *et al*. (2012) also reported that the mortality of larvae of *Ae*. *aegypti* on exposure to the essential oils of *Mentha piperita*, *Ocimum basilicum*, *Curcuma longa* and *Zingiber officinale* was dose-dependant.

The symptoms in larvae treated with the hexane and dichloromethane extracts of *Z. officinale* var. *rubrum, Z. montanum, Z. spectabile*, Z. *zerumbet* and *C. aeruginosa* were dependent on dosage and affected by different periods of exposure. The symptoms observed showed abnormal behaviours such as restlessness, sluggishness, and coiling movement, and subsequently settled at the bottom of the cup with abnormal wagging, tremors, convulsions, and paralysis, and later died slowly. Similar symptoms were clearly seen on larvae of *Cx*. *quinquefasciatus* after being tested with ethanolic extract of *Kaempferia galanga* (Insun *et al*., 1999). Ahmad *et al*. (2016) also reported that time

duration and concentration of extract caused abnormal behaviours of the larvae of *Ae. aegypti* and *Ae. albopictus* after exposure to the methanol extract of seaweeds.

5.2.2 Effect of the lethal concentration in killing the larvae of *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*

The high rates of larval mortality observed at the higher concentrations (200 and 300 mg/L of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa*, respectively) within a 24-h exposure implicate the high toxicity of the extract. Lethal concentration 50 (LC_{50}) value is the concentration at which 50% of the larvae were immobilized and lethal concentration 90 (LC_{90}) value is the concentration at which 90% of the larvae were immobilized.

According to the study by Komalamisra *et al*. (2005), the extracts with 50 mg/L<LC_{50} <100 mg/L were considered moderately effective and those with LC_{50}<50 mg/L were considered to be highly effective. Therefore, according to the classification of LC_{50} values by Komalamisra *et al*. (2005), 3 extracts were moderately effective against the larvae of *Ae. albopictus* that is the hexane extracts of *Z. officinale* var. *rubrum* (HZOR, LC_{50}= 96.86 mg/L), *Z. montanum* (HZM; LC_{50}= 99.04 mg/L) and *Z. spectabile* (HZS, LC_{50}= 93.51 mg/L); the hexane extracts of *Z. montanum* and *Z. zerumbet* (HZM and HZZ) also moderately effective against the larvae of *Ae. aegypti* (LC_{50}= 84.95 mg/L and 82.05 mg/L, respectively); and the hexane and dichloromethane extracts of *Z. zerumbet* and *C. aeruginosa* (HZZ, DZZ, HCA and DCA) were highly effective against the larvae of *Cx. quinquefasciatus* with LC_{50} values of 49.28, 30.15, 21.94, and 42.47 mg/L, respectively. Out of the nine extracts stated above which were found to be effective against the three mosquito species, seven of them were reported from the hexane extracts. This result is supported by Traboulsi *et al*. (2002) which stated that non-polar phyto products such as hexane extract from plants possessed high larvicidal activity. Variations in the results

obtained in the lethal concentration is probably due to the differences in levels of toxicity among the insecticidal ingredients of each plant (Manjari *et al*., 2014).

The results of this study are also comparable to earlier reports. The LC_{50} values of the hexane and dichloromethane extracts of five Zingiberaceae species in this study against *Ae*. *albopictus* larvae varies from 93.51 – 206.93 mg/L. This value is better than those reported by Vinayachandra *et al*. (2011) who examined the hexane extract of the kernel of *Knema attenuata* against the same mosquito species (LC_{50} value of 239 mg/L).

Result of the hexane extract of *Zingiber zerumbet* against *Cx*. *quinquefasciatus* larvae (LC_{50}= 49.28 mg/L) in the present study showed lower LC_{50} value than obtained by Kamaraj *et al*. (2010) who investigated the larvicidal activity of hexane extract of *Zingiber zerumbet*, ethyl acetate extract of *Dolichos biflorus* and methanol extract of *Aristolochia indica* against *Cx. quinquefasciatus* (LC_{50} = 69.18, 34.76, and 25.60 mg/L; LC_{90} = 324.40, 172.78, and 105.52 mg/L). This indicate that the hexane extract of *Z. zerumbet* in the present study was slightly effective than study by Kamaraj *et al*. (2010). It is probably because of the different geographical location where the plant species is grown (Das *et al*., 2007).

Warikoo *et al*. (2012) studied the larvicidal activities of *Ae*. *aegypti* using the hexane leaf extract of *Citrus sinensis*. The study found that the hexane leaf extracts exhibited moderate larvicidal efficiency with LC_{50} and LC_{90} values of 446.84 and 1,370.96 mg/L, respectively after 24 h exposure. In comparison, the larvicidal activity of hexane extracts of five Zingiberaceae species in the present study against *Ae*. *aegypti* larvae (LC_{50} and LC_{90} values ranged from 82.05–315.79 mg/L) showed relatively better values than obtained by Warikoo *et al*. (2012).

Dichloromethane extract of *Z. montanum* (DZM) against *Ae. aegypti* larvae (LC_{50}= 197.41 mg/L) in this study showed higher LC_{50} value than that obtained by Bandara *et al*. (2005) who studied a similar plant extract (dichloromethane extract of *Z. montanum*) against *Ae. aegypti* larvae (LC_{50} 4.76 mg/L). Anees (2008) have reported that the highest larval mortality of *Ae. aegypti* and *Cx. quinquefasciatus* were 150.40 ppm and 76.61 ppm found in chloroform and hexane extract of *Ocimum sanctum*. The results obtained by Anees (2008) was higher than those in the present study where the highest larval mortality of *Ae. aegypti* and *Cx. quinquefasciatus* larvae were recorded at 82.05 mg/L and 21.94 mg/L, respectively, in the hexane extracts of *Z. zerumbet* and *C. aeruginosa*.

The overall results in this study indicate a quite high larvicidal activity against the three mosquito species probably due to the mixture of chemical constituents that are present in the extracts. The chemical compounds such as zingerone, 1,1'-ethylenebisdecalin, zerumbone, trans-dihydrocarvone, carbendazim, terpinen-4-ol, zingiberene, β-sesquiphellandrene, camphene, α-caryophyllene, tropolone and camphor which were found in the hexane and dichloromethane extracts of five Zingiberaceae species may be responsible for this relatively high larvicidal activity since these chemicals have been reported to possess toxicity to many insects. However, minor constituents or other constituents that do not cause mortality, can produce in significant effect if these compounds are mixed (Hummelbrunner & Isman, 2001). Hence, other minor compounds such as zingerone, gingerol, α-curcumene, eucalyptol, humulene oxide and etc identified in the hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* should not be neglected.

The crude extracts may be more effective compared to the individual active compounds, due to natural synergism that discourages the development of resistance in the vectors (Veni *et al*., 2016). Even using the same phytochemicals, different

susceptibilities of the mosquito larvae was detected in this study. This was probably because of the differences in mosquito species and body size, sugar water availability, adult density in test cages, age of mosquito (Veni *et al*., 2016; Pitasawat *et al*., 2003), the variation in the plant species/locality of the source material which were influenced by different stress factor, such as soil temperature, soil type, pressure and overall climate of the area (Sukumar *et al*., 1991), the parts of the plant, the geographical location where the plants are grown and the application method (Das *et al*., 2007).

5.3 ADULTICIDAL ACTIVITY OF THE HEXANE AND DICHLOROMETHANE EXTRACTS OF FIVE ZINGIBERACEAE SPECIES AGAINST *AE. ALBOPICTUS*, *AE. AEGYPTI* AND *CX. QUINQUEFASCIATUS*

Based on the statistical analysis, the adulticidal activity of hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* against *Ae. aegypti* and *Cx. quinquefasciatus* were low for 24 h exposure whereas the adulticidal activity of the hexane extracts of *C. aeruginosa*, *Z. spectabile* and *Z. zerumbet* against *Ae. albopictus* showed higher mortality results (37.78%, 24.44% and 20.00%, respectively) than the other hexane and dichloromethane extracts for 24 h exposure. Similar to the larval stage, mortality of adult mosquitoes also increased with the increasing of exposure time (the regression equations maintain the formula of straight line) which is signified by the values of R in each case.

Hafeez *et al*. (2010) reported that the adulticidal activity of ten citrus oils was moderate to highly effective against *Ae. albopictus* with 45% to 82% mortality recorded. On the contrary, the adulticidal activity of *Ae. albopictus* in the present study was slightly less efective (<40% mortality) after being exposed with the hexane and dichloromethane extracts of five Zingiberaceae species as compared to the study by Hafeez *et al*. (2010).

Choochote *et al*. (2005) studied the adulticidal activity of *Curcuma aromatica* hexane extract against *Ae*. *aegypti* mosquito and found that this extract was effective with more than 95.00% mortality recorded. High mortality of *Ae*. *aegypti* was also recorded in the hexane and dichloromethane fractions of *Spondias mombin* (85.30% and 100.00%) as reported by Ajaegbu *et al*. (2016). According to these results, hexane extract of *Curcuma aromatica*, hexane and dichloromethane fractions of *Spondias mombin* were more effective against *Ae*. *aegypti* as compared to the hexane and dichloromethane extracts of *Z*. *officinale* var. *rubrum*, *Z*. *montanum*, *Z*. *spectabile*, *Z*. *zerumbet* and *C*. *aeruginosa* in the present study (< 10.00% mortality).

Elango *et al*. (2012) found that the adulticidal activity of acetone extracts of *A*. *marmelos*, hexane extract of *A*. *lineata*, ethyl acetate extract of *A*. *paniculata*, and methanol extracts of C. *hirsutus*, *E*. *prostrata*, and *T*. *erecta* against *Culex tritaeniorhynchus* was very high (100.00% died). Similarly, study by Kamaraj *et al*. (2010) found that the hexane and ethyl acetate extracts of *Zingiber zerumbet* also exhibited 100.00% mortality of *Culex gelidus* and *Cx*. *quinquefasciatus*. The results from Elango *et al*. (2012) and Kamaraj *et al*. (2010) as stated above showed that their extracts were more effective than the hexane and dichloromethane extracts of five Zingiberaceae species in the present study (<10.00% mortality) against *Cx*. *quinquefasciatus*.

Some of the plant extracts did not show any effective adulticidal activity as reported by Lee and Chiang (1994) but the larvicidal activity of *Stemona tuberosa* was good although no adulticide was detected. Choochote *et al*. (1999) also investigated the adulticidal activity of hexane fraction of *Kaempferia galanga* against *Cx*. *quinquefasciatus*, however the result showed that the extract only caused a knockdown effect at the initial stage of exposure but after transferring to the holding tube, the mosquito recovered from the knockdown effect. Then they concluded perhaps *K*. *galanga*

which belong to the family Zingiberaceae (aromatic plants), might be useful as a repellent instead. The other study conducted by Prajapati *et al*. (2005) also found that the essential oil of *Zingiber officinale* (Zingiberaceae) showed high ovicidal activity but no adulticidal activity against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. Since the species used in the present study are also from the same family with the species tested by Choocote *et al*. (1999) and Prajapati *et al*. (2005), it may be possible that the hexane and dichloromethane extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* were also useful for the repellency and ovicidal activity.

5.4 EFFECT OF THE HEXANE AND DICHLOROMETHANE EXTRACTS OF FIVE ZINGIBERACEAE SPECIES ON REPELLENCY, BLOOD-FEEDING INHIBITION AND MORTALITY ACTIVITY AGAINST *AE. ALBOPICTUS*, *AE. AEGYPTI* AND *CX. QUINQUEFASCIATUS* USING THE TUNNEL TEST

There are only a few published studies using the tunnel test method for repellency against mosquitoes. In the tunnel experiments, the feeding inhibition, repellence effect and mortality response measured on *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* were conducted at the concentration of 1000 mg/m^2. After 30 minutes, 1 h and 3 h exposure, 100% repellency was observed against the three species of mosquitoes in HZOR, DZOR, HZS and HCA. However, after 24 h exposure, the highest repellency against *Ae*. *albopictus* was recorded in DCA (50.67%), against *Ae*. *aegypti* was in HZM (89.33%) and against *Cx*. *quinquefasciatus* was in DZS (80.00%). Results on the repellency activity of the hexane and dichloromethane extracts of five Zingiberaceae species in the present study against *Ae*. *aegypti* showed relatively better activity (84.00% to 100%) after 3 h exposure than the study by Mukandiwa *et al*. (2016) who reported average repellency of 46.89% and 50.13% against *Ae*. *aegypti* in acetone and hexane extracts of *Clausena anisata*, respectively, at the same hour of exposure.

There was blood-feeding inhibition of 6.67% for *Ae*. *albopictus* and *Cx*. *quinquefasciatus* in DZZ and 12.00% for *Ae*. *aegypti* in DZOR after being exposed for 30 min. The highest blood-feeding inhibition against *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* after 1 h exposure were reported in DZZ, DZOR and DCA with 13.33%, 16.00% and 4.00%, respectively. *Ae*. *albopictus* in HZS (20.00%) and *Ae*. *aegypti* in DZOR (22.67%) showed higher blood-feeding inhibition than *Cx*. *quinquefasciatus* in DZZ (6.67%) after 3 h exposure. However, after 24 h exposure, *Ae*. *albopictus* in DZS showed the highest blood-feeding inhibition with 69.33%, followed by *Ae*. *aegypti* and *Cx*. *quinquefasciatus* in DZOR and DZZ (53.33%). Permethrin, a commonly used pyrethroid insecticide evaluated at dosage of 500 mg/m^2 in tunnel experiments against *Anopheles gambiae* and *Cx*. *quinquefasciatus* inhibit 100% blood-feeding activity (Hougard *et al*., 2003). In comparison to the study by Hougard *et al*. (2003), the present result of the hexane and dichloromethane extracts of five Zingiberaceae species showed weak blood-feeding inhibition towards *Cx*. *quinquefasciatus* (26.67%).

The mortality rates induced by hexane and dichloromethane extracts was very low (1.33%) for *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* after 30 min, 1 h and 3 h exposure. At the end of 24 h exposure, the highest mortality was observed against *Ae*. *albopictus* in HZS and DZZ (29.33%), against *Ae*. *aegypti* in DZZ (20.00%) and against *Cx*. *quinquefasciatus* in HCA (17.33%). Kweka *et al*. (2008) investigated the essential oils of *Ocimum suave* and *Ocimum kilimandscharicum* against the malaria vector, *Anopheles arabiensis*, *Anopheles gambiae* and *Culex quinquefasciatus*, in north-eastern Tanzania by tunnel test experiment. They found that the most effective dosage was 500 mg/m^2 and at this dosage, 50% to 67% mortality was recorded against three species of mosquitoes. Corbel *et al*. (2004) evaluated the permethrin (pyrethroid insecticides) against *Anopheles gambiae* s.s using tunnel test experiments. The result revealed that around 80% mortality was reported at the concentration of 500 mg/m^2. In contrast, less

than 15% mortality of *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* was obtained in the present study after being tested with the hexane and dichloromethane extracts of five Zingiberaceae species at the dosage of 500 mg/m^2. Therefore, these hexane and dichloromethane extracts were less effective in inhibiting *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* than study by Kweka *et al*. (2008) and Corbel *et al*. (2004).

Based on the tunnel test results, *Ae*. *albopictus* showed less repellency but high blood-feeding inhibition and mortality rate after being tested with the hexane and dichloromethane extracts of five Zingiberaceae species. In contrast, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* showed high repellency but low blood-feeding inhibition and mortality rates. The variation in the percentage repellency, blood-feeding inhibition and mortality of three mosquitoes among the extracts are probably due to the effect of the major active ingredients found in the extracts, the differences in plant species, part of the plant used, mosquito species and their behaviour, geographical varieties, extraction method as well as the polarity of the solvents used during extraction, biochemical attractiveness to biting mosquitoes, temperature, humidity and wind speed (Ghosh *et al*., 2012; Odalo *et al*., 2005; Phasomkusolsil & Soonwera, 2010; Beng *et al*., 2014).

Notably, the effect of the hexane and dichloromethane extracts of *Z*. *officinale* var. *rubrum*, *Z*. *montanum*, *Z*. *spectabile*, *Z*. *zerumbet* and *C*. *aeruginosa* vary depending on concentrations, exposure time and mosquito species. Overall, the hexane and dichloromethane extracts of five Zingiberaceae species exhibited higher larvicidal and repellency activity than adulticidal activity. Hence this study will lead to new knowledge in developing an ecofriendly control agents against the vector of dengue, chikungunya and Japanese encephalitis.

CHAPTER 6: CONCLUSION

In the present study, the insecticidal activity of the hexane and dichloromethane rhizome extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus* have been successfully conducted. The hexane and dichloromethane extracts of five Zingiberaceae species showed good results on larvicidal activity of *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus*.

The effective concentrations for larvicidal activity ranged from 21.94 mg/L to 99.04 mg/L. The hexane extracts of *Z. officinale* var. *rubrum* (96.86 mg/L), *Z. montanum* (99.04 mg/L) and *Z. spectabile* (93.51 mg/L) can be good plant candidates to control the larvae of *Ae*. *albopictus* whereas the hexane extracts of *Z. montanum* (84.95 mg/L) and *Z. zerumbet* (82.05 mg/L) were effective to control the larvae of *Ae*. *aegypti.* The hexane and dichloromethane extracts of *Z. zerumbet* (49.28 mg/L and 30.15 mg/L) and *C. aeruginosa* (21.94 mg/L and 42.47 mg/L) were highly effective in killing *Cx. quinquefasciatus* larvae. The percentage mortality of the larvae was increased when the concentrations and exposure time increased.

In adulticidal bioassays, exposure time contributed to the increase in the mortality number of the adult mosquitoes in all five Zingiberaceae species extracts. Low adulticide effects (37.78%) towards *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* was reported at the concentration of 1000 mg/m^2. Since the concentration of 1000 mg/m^2 showed less effective for adulticidal activity, therefore, it was not recommended for this study and suggested to use higher concentration. The most effective extract to kill *Ae*.

albopictus was HCA (37.78%), for *Ae*. *aegypti* was DZOR (6.67%), and for *Cx*. *quinquefasciatus* was HCA and DZZ (2.22%).

The results from the tunnel test showed that among the three mosquito species, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* showed high repellency activity as compared to the moderate repellency activity against *Ae*. *albopictus*. The highest repellency activity against *Ae*. *albopictus* was reported in dichloromethane extract of *C*. *aeruginosa* (50.67%), against *Ae*. *aegypti* was in hexane extract of *Z*. *montanum* (89.33%), and against *Cx*. *quinquefasciatus* was in dichloromethane extract of *Z*. *spectabile* (80.00%).

Moderate blood-feeding inhibition of *Ae*. *albopictus*, *Ae*. *aegypti* and *Cx*. *quinquefasciatus* were identified at the end of 24 h exposure in all five Zingiberaceae species extracts. Dichloromethane extract of *Z*. *spectabile* was shown as the most effective extract in inhibiting *Ae*. *albopictus* (69.33%), dichloromethane extract of *Z*. *officinale* var. *rubrum* for *Ae*. *aegypti* (53.33%) and dichloromethane extract of *Z*. *zerumbet* for *Cx*. *quinquefasciatus* (53.33%).

Mortality rates of the three mosquito species after being tested with the hexane and dichloromethane extracts of five Zingiberaceae species was low (< 29.33%) after 24 h exposure. *Ae*. *aegypti* and *Cx*. *quinquefasciatus* were more resistant than *Ae*. *albopictus*. Hexane extract of *Z*. *spectabile* induced high mortality of *Ae*. *albopictus* (29.33%), dichloromethane extract of *Z*. *zerumbet* also induced high mortality of *Ae*. *albopictus* (29.33%) as well as *Ae*. *aegypti* (20.00%), and hexane extract of *C*. *aeruginosa* induced high mortality of *Cx*. *quinquefasciatus* (17.33%).

Therefore, the results reported in this present study showed that the hexane and dichloromethane extracts of *Z*. *officinale* var. *rubrum*, *Z*. *montanum*, *Z*. *spectabile*, *Z*. *zerumbet* and *C*. *aeruginosa* have potential as larvicide and as repellent than as an

adulticide. However, the hexane and dichloromethane extracts of five Zingiberaceae species used in this study can still be good candidates in integrated vector control program. Thus, further study is needed in order to find out the active compound/s from the hexane and dichloromethane extracts of five Zingiberaceae species that responsible for the insecticidal activity. Future research on the essential oils of Z. *officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *C. aeruginosa* should also be carried out that is responsible as bio-insecticide.

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LIST OF PUBLICATION AND PAPERS PRESENTED

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Efficacy of Four Species of Zingiberaceae Extract Against Vectors of Dengue, Chikungunya and Filariasis

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Abstract. Laboratory bioassays on insecticidal activity of hexane crude extract derived from four species of *Zingiber*: *Zingiber officinale* var. *rubrum* (HZOR), *Zingiber montanum* (HZM), *Zingiber spectabile* (HZS) and *Zingiber zerumbet* (HZZ) were carried out against three mosquito larvae vector: *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus*. GC/MS analysis revealed 43, 82, 50, and 51 compounds from HZO, HZM, HZS, and HZZ, respectively. The major principal constituents found in HZO extract were zingerone (14.92%) and benzaldehyde dimethyl thiol acetal (11.61%); HZM extract were dimethyl 4-methylphthalate (12.64%) and carbendazim (12.62%); HZS extract had 1,1'-ethylenebisdecalin (42.52%) and 1-pentadecyne (11.5%); and HZZ extract were humulene epoxide II (20.84%) and zerumbone (60.4%). Assessment of larvicidal efficacy demonstrated good larvicide effects towards all the crude hexanes. The mortality was observed after 24h exposure. The highest larvicidal mortality of *Ae. albopictus* larvae was found in HZOR, HZM, and HZS (LC_{50}= 96.86, 99.04 and 93.35 mg/L; LC_{90}= 168.65, 153.77, and 168.65 mg/L) respectively. HZM and HZZ were effective against *Ae. aegypti* larvae with LC_{50}= 84.95 and 82.05 mg/L and LC_{90}= 134.85 and 121.05 mg/L, respectively. HZZ showed the most effective extract against *Cx. quinquefasciatus* larvae with LC_{50}= 49.28 mg/L and LC_{90}= 83.87 mg/L. No mortality was recorded in the control. Results from studies suggest that bioassay-guided effective extracts of *Z. officinale* var. *rubrum*, *Z. montanum*, *Z. spectabile* and *Z. zerumbet* are potential larvicidal candidates for controlling *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus*.

INTRODUCTION

Over 2 billion people in the tropics have been infected by mosquito-borne diseases, such as malaria, chikungunya, dengue fever, lympatic filariasis, yellow fever, Japanese encephalitis etc. (Govindarajan *et al.*, 2011). *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* are three medically important vectors in Malaysia. *Aedes* species are known as vectors transmitting dengue and chikungunya virus, while *Cx. quinquefasciatus* is a vector that transmits Japanese encephalitis (Vinayachandra *et al.*, 2011).

Chemical control using synthetic insecticides have been used so far and it was favourable because of their speedy action and easy to employ. However, the major problems using the chemicals for controlling the mosquitoes are the development of resistance by the mosquito, damaging environment and also human (Rahuman *et al.*, 2008). Fortunately, plants which are rich in bioactive chemicals probably can be alternative insecticide to control the mosquitoes. Because of this, much effort has been focused on plant extracts or phytochemicals as potential sources of commercial mosquito control agents for the interruption of the transmission of mosquito-borne diseases at the individual as well as at the community level (Govindarajan *et al.*, 2011).

APPENDICES

Appendix A: Example records of WHO larvicidal bioassay on the total mortality of *Ae. albopictus* in the hexane and dichloromethane extracts of five Zingiberaceae species at different exposure time

Solvents	Zingiberaceae species	Exposure time and concentrations (mg/L)																			
		1 h					3 h					6 h					24 h				
		Control	70	100	200	300	Control	70	100	200	300	Control	70	100	200	300	Control	70	100	200	300
Hexane	*Z. officinale* var. *rubrum*	0	0	0	3	10	0	8	6	35	64	0	16	15	60	75	0	25	27	74	75
	Z. montanum	0	0	0	1	2	0	0	0	21	40	0	0	2	45	67	0	16	31	75	75
	Z. spectabile	0	0	0	0	1	0	0	1	0	6	0	3	3	6	36	0	24	36	72	75
	Z. zerumbet	0	0	0	0	1	0	0	0	3	12	0	0	0	23	52	0	13	22	75	75
	C. aeruginosa	0	0	0	0	0	0	0	0	0	9	0	0	1	1	17	0	5	3	50	75
*DCM	*Z. officinale* var. *rubrum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	33	67
	Z. montanum	0	0	0	0	0	0	0	0	1	19	0	0	1	4	29	0	0	5	28	69
	Z. spectabile	0	0	0	0	0	0	0	0	2	13	0	1	2	3	34	0	7	10	61	75
	Z. zerumbet	0	0	0	0	0	0	0	0	2	16	0	0	0	8	39	0	3	3	68	75
	C. aeruginosa	0	0	0	0	0	0	0	0	5	57	0	0	0	14	63	0	1	2	67	75

*DCM= dichloromethane

Appendix B: Example of print out of Generalize Linear Model against larvae of *Ae. albopictus* after being tested with the hexane extracts of five Zingiberaceae species

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	7699.24	1	0.000
ZingiberaceaeSp	989.8	4	0.000
Time	5733.269	3	0.000
conc	3798.889	3	0.000
ZingiberaceaeSp * Time	598.985	12	0.000
ZingiberaceaeSp * conc	380.38	12	0.000
Time * conc	1928.217	9	0.000
ZingiberaceaeSp * Time * conc	569.424	36	0.000

Dependent Variable: no of mortality
Model: (Intercept), ZingiberaceaeSp, Time, conc, ZingiberaceaeSp * Time, ZingiberaceaeSp * conc, Time * conc, ZingiberaceaeSp * Time * conc

Pairwise Comparisons

(I) Zingiberaceae Sp.	(J) Zingiberaceae Sp.	Mean Difference (I-J)	Std. Error	df	Sig.	95% Wald Confidence Interval for Difference	
						Lower	Upper
HZOR	HZM	2.458[a]	0.235	1	0.000	1.997	2.92
	HZS	4.792[a]	0.235	1	0.000	4.33	5.253
	HZZ	4.521[a]	0.235	1	0.000	4.059	4.982
	HCA	6.917[a]	0.235	1	0.000	6.455	7.378
HZM	HZOR	-2.458[a]	0.235	1	0.000	-2.92	-1.997
	HZS	2.333[a]	0.235	1	0.000	1.872	2.795
	HZZ	2.063[a]	0.235	1	0.000	1.601	2.524
	HCA	4.458[a]	0.235	1	0.000	3.997	4.92
HZS	HZOR	-4.792[a]	0.235	1	0.000	-5.253	-4.33
	HZM	-2.333[a]	0.235	1	0.000	-2.795	-1.872
	HZZ	-0.271	0.235	1	0.250	-0.732	0.191
	HCA	2.125[a]	0.235	1	0.000	1.664	2.586
HZZ	HZOR	-4.521[a]	0.235	1	0.000	-4.982	-4.059
	HZM	-2.063[a]	0.235	1	0.000	-2.524	-1.601
	HZS	0.271	0.235	1	0.250	-0.191	0.732
	HCA	2.396[a]	0.235	1	0.000	1.934	2.857
HCA	HZOR	-6.917[a]	0.235	1	0.000	-7.378	-6.455
	HZM	-4.458[a]	0.235	1	0.000	-4.92	-3.997
	HZS	-2.125[a]	0.235	1	0.000	-2.586	-1.664
	HZZ	-2.396[a]	0.235	1	0.000	-2.857	-1.934

Pairwise comparisons of estimated marginal means based on the linear predictor of dependent variable no of mortality

[a] The mean difference is significant at the .05 level.

Pairwise Comparisons

(I) exposure time (h)	(J) exposure time (h)	Mean Difference (I-J)	Std. Error	df	Sig.	95% Wald Confidence Interval for Difference	
						Lower	Upper
1 h	3 h	-3.117[a]	0.211	1	0.000	-3.529	-2.704
	6 h	-6.733[a]	0.211	1	0.000	-7.146	-6.321
	24 h	-15.083[a]	0.211	1	0.000	-15.496	-14.671
3 h	1 h	3.117[a]	0.211	1	0.000	2.704	3.529
	6 h	-3.617[a]	0.211	1	0.000	-4.029	-3.204
	24 h	-11.967[a]	0.211	1	0.000	-12.379	-11.554
6 h	1 h	6.733[a]	0.211	1	0.000	6.321	7.146
	3 h	3.617[a]	0.211	1	0.000	3.204	4.029
	24 h	-8.350[a]	0.211	1	0.000	-8.763	-7.937
24 h	1 h	15.083[a]	0.211	1	0.000	14.671	15.496
	3 h	11.967[a]	0.211	1	0.000	11.554	12.379
	6 h	8.350[a]	0.211	1	0.000	7.937	8.763

Pairwise comparisons of estimated marginal means based on the linear predictor of dependent variable no of mortality

[a] The mean difference is significant at the .05 level.

Pairwise Comparisons

(I) concentration (mg/L)	(J) concentration (mg/L)	Mean Difference (I-J)	Std. Error	df	Sig.	95% Wald Confidence Interval for Difference	
						Lower	Upper
70	100	-.617[a]	0.211	1	0.003	-1.029	-0.204
	200	-7.233[a]	0.211	1	0.000	-7.646	-6.821
	300	-10.950[a]	0.211	1	0.000	-11.363	-10.537
100	70	.617[a]	0.211	1	0.003	0.204	1.029
	200	-6.617[a]	0.211	1	0.000	-7.029	-6.204
	300	-10.333[a]	0.211	1	0.000	-10.746	-9.921
200	70	7.233[a]	0.211	1	0.000	6.821	7.646
	100	6.617[a]	0.211	1	0.000	6.204	7.029
	300	-3.717[a]	0.211	1	0.000	-4.129	-3.304
300	70	10.950[a]	0.211	1	0.000	10.537	11.363
	100	10.333[a]	0.211	1	0.000	9.921	10.746
	200	3.717[a]	0.211	1	0.000	3.304	4.129

Pairwise comparisons of estimated marginal means based on the linear predictor of dependent variable no of mortality

[a] The mean difference is significant at the .05 level.

Appendix C: Example records of WHO adult bioassay on the total mortality of *Ae. albopictus* in the hexane and dichloromethane extracts of five Zingiberaceae species at different exposure time

Solvents	Mosquito species	Zingiberaceae species	Exposure time			
			1 h	2 h	3 h	24 h
Hexane	*Ae. albopictus*	*Z. officinale* var. *rubrum*	1	1	1	3
		Z. montanum	0	0	0	0
		Z. spectabile	0	0	4	11
		Z. zerumbet	0	1	4	9
		C. aeruginosa	1	4	9	17
	Ae. aegypti	*Z. officinale* var. *rubrum*	0	0	0	1
		Z. montanum	0	0	0	0
		Z. spectabile	0	0	0	1
		Z. zerumbet	0	0	0	2
		C. aeruginosa	0	0	0	0
	Cx. quinquefasciatus	*Z. officinale* var. *rubrum*	0	0	0	0
		Z. montanum	0	0	0	0
		Z. spectabile	0	0	0	0
		Z. zerumbet	0	0	0	0
		C. aeruginosa	0	0	1	1
	Control		0	0	0	0
*DCM	*Ae. albopictus*	*Z. officinale* var. *rubrum*	0	0	0	0
		Z. montanum	0	0	0	0
		Z. spectabile	0	0	1	7
		Z. zerumbet	0	1	1	3
		C. aeruginosa	0	0	0	0
	Ae. aegypti	*Z. officinale* var. *rubrum*	0	0	0	3
		Z. montanum	0	0	0	0
		Z. spectabile	0	0	0	0
		Z. zerumbet	0	0	1	1
		C. aeruginosa	0	0	0	2
	Cx. quinquefasciatus	*Z. officinale* var. *rubrum*	0	0	0	0
		Z. montanum	0	0	0	0
		Z. spectabile	0	0	0	0
		Z. zerumbet	0	0	1	1
		C. aeruginosa	0	0	0	0
	Control		0	0	0	0

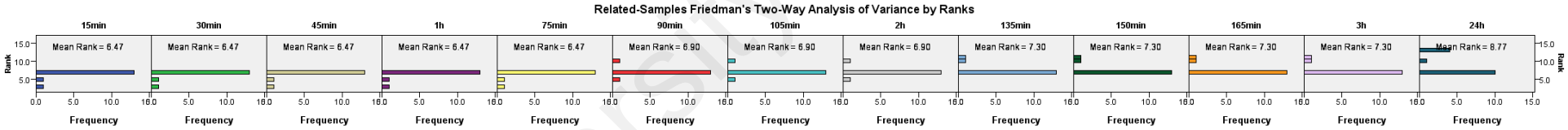
*DCM= dichloromethane

Appendix D: Example of print out of Related-samples Friedman's Two-way Analysis of variance by Ranks against adult mosquito of *Ae. albopictus* in the dichloromethane extracts of five Zingiberaceae species

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of Time 1, Time 2, Time 3, Time 4, Time 5, Time 6, Time 7, Time 8, Time 9, Time 10, Time 11, Time 12 and Time 13 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.



Total N	15
Test Statistic	34.869
Degrees of Freedom	12
Asymptotic Sig. (2-sided test)	.000

Appendix E: Total of blood-feeding inhibition, repellency and mortality activity of adult *Ae*. *albopictus* in the hexane and dichloromethane extracts of five Zingiberaceae species

Zingiberaceae species	Solvents	Exposure time	Tot_blood-feedinginhibition	Tot_repel	Tot_mort
Z. officinale var. *rubrum*	Hexane	30 min	0	75	0
		1 h	5	70	0
		3h	7	68	0
		24 h	38	28	9
	*DCM	30 min	0	75	0
		1 h	0	75	0
		3h	0	75	0
		24 h	23	35	17
	Control	30 min	12	66	0
		1 h	21	60	0
		3h	24	57	0
		24 h	53	19	2
Z. montanum	Hexane	30 min	2	73	0
		1 h	3	72	0
		3h	9	65	1
		24 h	28	37	10
	*DCM	30 min	2	73	0
		1 h	1	74	0
		3h	1	74	0
		24 h	31	35	9
	Control	30 min	21	72	0
		1 h	32	69	0
		3h	38	69	0
		24 h	57	20	0
Z. spectabile	Hexane	30 min	4	71	0
		1 h	2	73	0
		3h	15	60	0
		24 h	32	21	22
	*DCM	30 min	3	69	0
		1 h	5	70	0
		3h	6	69	0
		24 h	52	18	5
	Control	30 min	16	62	0
		1 h	24	56	0
		3h	30	48	0
		24 h	43	27	2

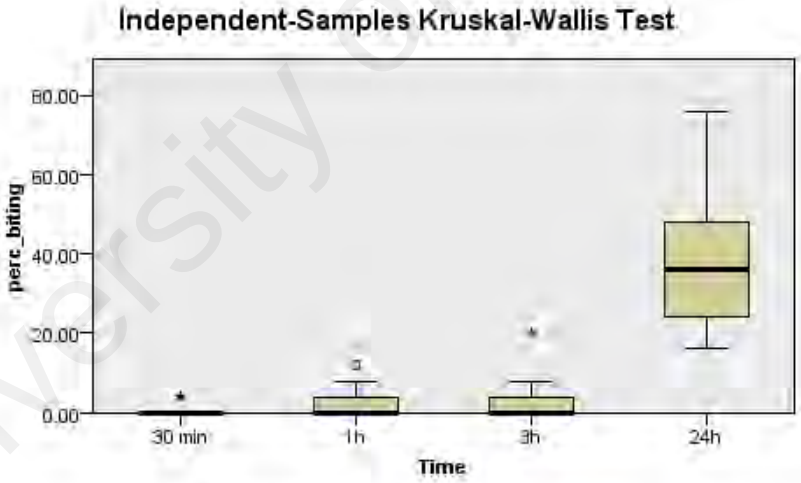
Appendix E, Continued

Zingiberaceae species	Solvents	Exposure time	Tot_blood-feedinginhibition	Tot_repel	Tot_mort
Z. zerumbet	Hexane	30 min	1	74	0
		1 h	2	73	0
		3h	2	73	0
		24 h	34	23	18
	*DCM	30 min	5	70	0
		1 h	10	65	0
		3h	13	62	0
		24 h	38	15	22
	Control	30 min	12	71	0
		1 h	15	66	0
		3h	21	64	0
		24 h	38	29	2
C. aeruginosa	Hexane	30 min	4	71	0
		1 h	4	71	0
		3h	5	70	0
		24 h	22	35	18
	*DCM	30 min	2	73	0
		1 h	2	73	0
		3h	1	74	0
		24 h	27	38	10
	Control	30 min	12	69	0
		1 h	15	66	0
		3h	18	58	0
		24 h	39	43	2

*DCM= Dichloromethane

Appendix F: Example of print out of Independent-Samples Kruskal –Wallis Test against the three adult mosquito species on the percentage of blood-feeding inhibition, repellency and mortality in the hexane extract of *Z. officinale* var. *rubrum* at different exposure time

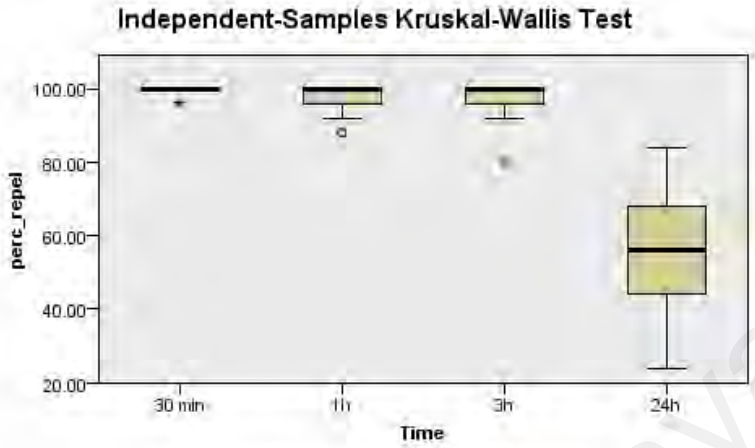
Hypothesis Test Summary				
	Null Hypothesis	**Test**	**Sig.**	**Decision**
1	The distribution of perc_blood-feeding is the same across categories of Time.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
2	The distribution of perc_repel is the same across categories of Time.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
3	The distribution of perc_mort is the same across categories of Time.	Independent-Samples Kruskal-Wallis Test	.001	Reject the null hypothesis.
Asymptotic significances are displayed. The significance level is .05.				



Total N	36
Test Statistic	22.450
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

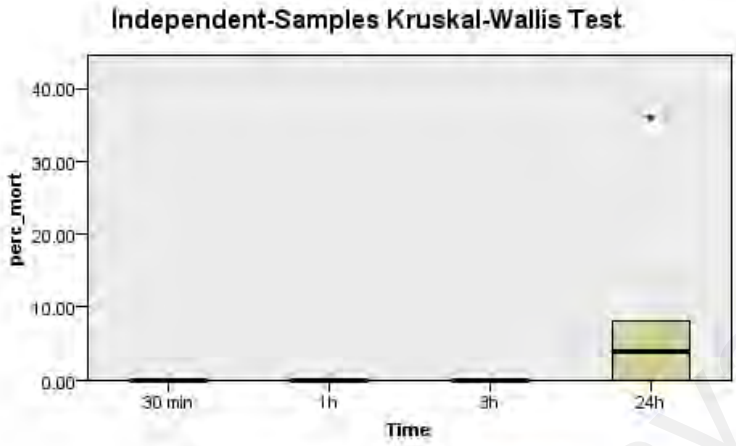
Appendix F, Continued



Total N	36
Test Statistic	22.610
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

Appendix F, Continued



Total N	36
Test Statistic	16.839
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.001

1. The test statistic is adjusted for ties.