HAZARD CLASSIFICATION OF GAMAT – *N,N*-DIETHYL-M-TOLUAMIDE (DEET) MOSQUITO REPELLENT CREAM USING THE UNITED NATIONS GLOBALLY HARMONIZED SYSTEM (UN-GHS) BASED ON RATS ACUTE DERMAL TOXICITY

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2015

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by

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Thesis submitted in the fulfillment of the requirements for the degree of Master of Science

AUGUST 2015

DECLARATION

I hereby declare that I am the sole author of this thesis in title "Hazard Classification of Gamat-*N*,*N*-diethyl-meta-toluamide (DEET) Mosquito Repellent Cream using the United Nations Globally Harmonized System (UN-GHS) based on Rats Acute Dermal Toxicity". I declare that the thesis is being submitted to Universiti Sains Malaysia (USM) for the purpose of the award of Master of Science in Health Toxicology. This thesis is the result of my own research under supervision of Dr. Jahangir bin Kamaldin except as cited in the references. The thesis has being accepted for the respective study and is not concurrently submitted in candidature of any other degree.

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ACKNOWLEDGEMENT

In the name of Allah, The Most Beneficent, The Most Merciful. Alhamdulillah, with His blessings, finally, I manage to complete my dissertation after the hard work, blood, sweat and tears spent during the whole study period. First and foremost, I would like to express my deep and sincere gratitude to my husband, Wan Khairul Anwar who is the main sponsor for this study, spiritual and financial. Without his sacrifice, I will not be able to complete this study.

Secondly, I would like to present my humble appreciation to my supervisor, Dr. Jahangir bin Kamaldin and co-supervisor, Dr. Nor Aini binti Saidin for their great effort to guide me and sharing the wonderful knowledge throughout the research. Also, I would like to thank my colleague, Izzati Abdul Malik who always share a bundle of information, assists me during the experiments as well as motivate me to the end of this work. May Allah bless you and your family.

I wish to thank my wonderful friends, all my postgraduate friends who always be my bread and butter, be together with me with laughter and tears. Allah's bless will always be with you guys and in sha Allah you will succeed here and hereafter. Last, but not least, thank you to my wonderful family for their encouragement and moral support during the production of this dissertation. Thank you Allah for Your wonderful gift. Alhamdulillah.

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LIST OF SYMBOLS AND ABBREVIATIONS

bw	body weight
DEET	*N, N*-diethyl-m-toluamide
EPA	Environmental Protection Agency
GC	Gamat Cream
GHS	Globally Harmonized System
kg	kilogram
mg	miligram
ml	mililiter
MRC	Mosquito Repellent Cream
OECD	Organization for Economic Cooperation and Development
SC	Stratum corneum
TG	Test Guideline
UN	United Nations

ABSTRAK

Kajian ini bertujuan mengenal pasti tanda-tanda klinikal yang dipamerkan oleh tikus apabila terdedah kepada produk Krim Gamat - *N*,*N*-diethyl-m-toluamide (DEET) Penghalau Serangga (GDMRC). Produk ini mengandungi *N*,*N*-diethyl-m-diethyl (15 percent berat per berat) sebagai bahan aktif dengan bioaktiviti menghalau nyamuk dan Krim Gamat (GC) sebagai bahan plasebo. Kajian ini dilakukan untuk menentukan kategori bahaya ketoksikan kulit akut bagi GDMRC seterusnya mengklasifikasikan ia berdasarkan Kelas Racun Bahaya WHO dan klasifikasi harmoni bahan kimia berbahaya UN-GHS. Data kajian menyediakan sebahagian daripada data keselamatan yang diperlukan untuk tujuan pendaftaran produk di mana melalui laluan pendedahan kulit adalah pasti kerana item ujian adalah bertujuan untuk permohonan topikal pada kulit manusia. Kajian ini menggunakan kaedah seperti yang dinyatakan dalam Garis Panduan Ujian OECD 402. Keputusan menunjukkan bahawa semua haiwan yang didedahkan sama ada dengan GDMRC atau GC sehingga hari ke 14 terselamat tanpa kematian, hampir menemui ajal, sakit teruk atau kesusahan yang teruk. Tiada tanda-tanda klinikal yang berterusan atau tidak berbalik, diperhatikan sepanjang 14 hari pemerhatian. Kesimpulannya, GDMRC memenuhi Kelas U dalam Kelas Ketoksikan Bahaya WHO dan Kategori 5 dalam Kategori Ketoksikan Akut Bahaya UN-GHS. Maka, untuk penggunaan biasa, GDMRC tidak mempunyai kebangkalian untuk mendatangkan bahaya akut kepada kulit.

ABSTRACT

The study is aimed to identify clinical signs exhibited by rats upon single exposure of Gamat – *N*,*N*-diethyl-m-toluamide (DEET) Mosquito Repellent Cream (GDMRC) containing 15 percent of *N*,*N*-diethyl-m-toluamide as the active ingredient with bioactivity (repellent) against mosquitoes which includes Gamat Cream (GC) as the placebo item. The study is performed to determine the hazard category of acute dermal toxicity of the GDMRC subsequently to classify it based on the WHO Pesticide Hazard Class and the UN-GHS harmonized classification of hazardous chemical substances. The study data provides a part of the safety data required for registration purpose of the test item, where exposure by the dermal route is definite because the test item is intended for human topical application on skin. The study employs method as described in the OECD Test Guideline 402. Results showed that all test animals dosed either with the GDMRC or the GC, survived until day-14 without mortality, moribund, severe pain or severe distress. There was no persistent or non-reversible clinical signs observed between day-1 until day-14.The study concludes that the GDMRC satisfies the hazard toxicity class of Class U based on WHO Recommended Classification of Pesticides by Hazard (WHO, 2010) and the Category 5 of Acute Toxicity Hazard Categories under the United Nations Globally Harmonized System for Classification of Chemical Substances and Mixtures (UN-GHS). Therefore, the GDMRC is unlikely to present acute hazard upon dermal contact under normal use.

CHAPTER 1

INTRODUCTION

Dengue is a tropical and subtropical mosquito-borne viral disease and has been a major health concern especially in Malaysia (Chew *et al.* 2012). It was estimated about 50 million dengue cases worldwide every year (WHO 2009). Dengue infection is related to seasons due to higher activity after monsoon. Dengue virus is spreads by *Aedes aegypti* and *Aedes albopictus* mosquito and develops dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).

DF and DHF have become a major health problem in Malaysia as the cases increased dramatically from year 1988 to 1998. Ministry of Health Malaysia reported that there were 26,240 cases of DF and 1,141 cases of DHF at the time of 1998 outbreak (Chew *et al*. 2012). Extensive development and urbanization created more breeding areas of mosquitoes and this resulted in higher incidence of dengue viral infections.

In view no vaccine, specific antidote and medications are available, preventive measure against dengue infection is crucial. Wearing protective clothing and application of insect repellent to the skin are the common approach in avoiding mosquitoes bites (Peter & Mwangi 2014). Two major types of commercial insect repellents available are synthetic chemicals and plant derived essential (Fradin & Day 2002). For the past 60 years, synthetic mosquito repellent, *N*,*N*-diethyl-meta-toluamide (DEET) was used worldwide (Isman & Grieneisen 2014). DEET is the most effective active ingredient for insect repellent (Moore & Debboun 2007) due to its longer protection time and inexpensive. Among commercial DEET formulations are aerosol/spray, creams, liquids,

roll-ons, towellettes, lotions, and impregnated wrist bands (Wilson *et al.* 2013).

In the study, Gamat-DEET Mosquito Repellent Cream (GDMRC) is an insect repellent product of cream formulation designed for topical application by end user at home. It is regarded as insecticide product and going to be marketed in Malaysia. In view the GDMRC via topical application causes direct exposure to human, the Malaysia Pesticide Board has mandated for any pesticide product to be registered for sale or distribution in Malaysia shall furnish non-clinical safety data (Pesticide Act 1974). Among the requirements for registration are safety data in form of acute dermal toxicity, acute oral toxicity, skin irritation and skin sensitization. Therefore, the study addresses acute dermal toxicity test. Acute dermal toxicity result will also be utilized in determining the hazard class of the insecticide product based on World Health Organization (WHO) Recommended Classification of Pesticides by Hazard as described in Table 1.1 (WHO 2010) that has been aligned with the Acute Toxicity Hazard Categories under the United Nations Globally Harmonized System for Classification of Chemical Substances and Mixtures (UN-GHS), whereby the GDMRC shall be only permitted for intended regular skin application if it meets the WHO Hazard Class U and the UN-GHS Category 4 and above.

The study specifically includes the cream base as the placebo item. The inclusion of the Gamat Cream (GC) as the placebo item is to verify any possible toxicity of the base formulation in view of none availability of prior safety data. Thus, the study is performed with the purpose to determine the acute dermal toxicity of GDMRC based on the harmonized UN-GHS toxic class with three main objectives which are:

1. To identify clinical signs exhibited by the rats upon single dosing of the test item (GDMRC) and the placebo item (GC), subsequently determining the

need for repeated dose toxicity study based on the outcome of the necropsy gross examination.

2. To determine the hazard category of the test item (GDMRC) based on the UN-GHS classification, subsequently concluding the suitability for sale under the group of household insecticide product which only accepts products with Hazard Category 4 and above.
3. To determine the hazard category of the placebo item (GC), to elucidate the possible toxicity contributed by the inert ingredients.

The outcome of the study shall provide a part of the safety data required for registration purpose of the test item (GDMRC) and shall provide information on health hazards likely to arise from a short term exposure (24 hours) to the test item by means of the dermal route. Therefore, the test item can be marketable under the group of household insecticide product in Malaysia and also in European Country in the future. In the other hand, this study also promotes the use of mosquito repellent among the society in order to prevent dengue cases.

Table 1.1 WHO Recommended Classification of Pesticides by Hazard

	WHO Pesticide Hazard Class	LD50 for the rat (mg/kg body weight)	
		Oral	Dermal
Ia	Extremely hazardous	< 5	< 50
Ib	Highly hazardous	5 – 50	50 – 200
II	Moderately hazardous	50 – 200	200 – 2000
III	Slightly hazardous	> 2000	> 2000
U	Unlikely to present acute hazard	≥ 5000	≥ 5000

(WHO 2010)

CHAPTER 2

LITERATURE REVIEW

2.1 United Nations Globally Harmonized System for Classification and Labeling of Chemical Substances and Mixtures (UN-GHS)

2.1.1 Purpose of UN-GHS

United Nations Globally Harmonized System for Classification and Labeling of Chemical Substances and Mixtures (UN-GHS) was developed at the Rio Summit in 1992. United Nation Economic and Social Council has adopted UN-GHS and endorsed by World Summit for Sustainable Development (WSSD) to be implemented in 2008. In view that the presence of multiple existing laws or regulations on chemical classification and labeling such as United Nations Dangerous Goods System, European Country (EC) system for hazardous substances (Winder *et al.* 2005) and in Malaysia, Chemical Packaging and Labeling (CPL) Regulation 1997 (OSHA 1994) have variations in definitions of hazards and caused confusion at user level, UN-GHS is purposely developed to standardize worldwide safe chemical management issues from manufacturer, transport, usage and disposal (Winder *et al.* 2005).

UN-GHS is an internationally agreed system for hazard classification and communication of chemical products (Pratt 2002) and was developed through a collaborative effort of various organizations such as WHO, International Labor Organization, Organization for Economic Cooperation and Development (OECD), United Nations, member countries of the organizations and support for chemical industries (Winder *et al.* 2005).

UN-GHS implementation is intended to provide an internationally comprehensible system for hazard communication, to provide a recognized framework for those countries without an existing system, to reduce the need for testing and evaluation of chemicals, and to facilitate international trade in chemicals whose hazards have been properly assessed and identified on an international basis (Pratt 2002).

The scope of the UN-GHS is based on the mandate from the 1992 United Nations Conference on Environment and Development (UNCED), which stated that hazard classification and labeling system of chemicals should be harmonized and compatible worldwide and feasible by the year 2000. Since then, UN-GHS was developed and improvised. The UN-GHS includes these two main elements which are harmonized criteria for classifying substances and mixtures according to their health, environmental, and physical hazards and harmonized hazard communication elements, including requirements for labeling and safety data sheets (UN-GHS 2011).

2.1.2 Hazard Classification

As an internationally agreed system for classification and labeling of chemical substances and mixtures, UN-GHS classified them in terms of physical hazard, health hazard and environmental hazard as described in Table 2.1 (UN 2011) which covers range of consumer products, workplace, pesticides and transportation. Implementation of UN-GHS does not only protect human health and environment but indirectly promote chemical trade between countries all over the world (Thannimalay & Yusoff 2008).

UN-GHS hazard classification includes the compilation of all relevant data need to be carried out by the supplier in order to place the item on the European Union market. In order to determine the hazard class, a cut off values for maximum levels is to

Table 2.1 UN-GHS Classification of Chemical Substance and Mixtures

Hazard Classification	Types of Chemical Substances and Mixtures
Physical hazard	Explosives
	Flammable gases
	Flammable liquids
	Flammable solids
	Oxidizing gases
	Oxidizing liquids
	Oxidizing solids
Health hazard	Corrosive to metals
	Skin corrosions
	Acute toxicity
	Skin irritants
	Carcinogenicity
Environmental hazard	Acute hazardous to the aquatic environment

(UN 2011)

be considered when evaluating test result of animal experiments. These cut-off values reflect a threshold between toxicological relevance and non-relevance to humans, whereby it covers all exposure situations presumably appearing under real-life conditions. Hence, a proper labeling is required to identify and specify the hazard class of the product (Gebel *et al.* 2009).

UN-GHS hazard communication is based on the provision of signal words, hazard statements and pictograms. All of these are connected to the specific hazard of the substances or mixtures (Silk 2003). Regulation (EC) No. 1907/2006 subjecting the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) entered into force in 2007 and represents a major piece of chemical legislation in Europe. One of the main objectives of the regulation is to ensure a maximum level of humans and environment protection. In achieving this objective, the chemical substances and mixtures are classified according to their physicochemical, environmental or human health hazards (Oltmanns *et al.* 2014). Based on the classification as described in Table 2.1, the study omitted acute toxicity that is under class of health hazard.

2.1.3 Health Hazards

Health hazard is defined as a substance for which there is statistically significant evidence based on at least one study conducted in accordance with established scientific principles that acute or chronic health effects may occur in exposed human. Health hazard chemicals include chemicals which are carcinogens, toxic or highly toxicagents, reproductive, toxins, irritants, corrosives, sensitizers, hepatotoxins, nephrotoxins, neurotoxins, agents which act on the hematopoietic system, and agents which damage the lungs, skin, eyes, or mucous membranes (UNECE 2003).

Health hazard classification covers criteria in terms of acute toxicity, skin and eye irritation/corrosion, danger of irreversible effects after single exposure and danger of serious damage to health by prolonged exposure (Gebel *et al.* 2009). Below are the summarizations of all the criteria that have been developed for the health hazard groupings (Winder *et al.* 2005; UN 2011):

- Single dose toxicity, covering a range of toxicity endpoints by various routes of exposure (Table 2.2).
- Skin irritation and corrosion. Category 1 is for corrosive effects and Categories 2 and 3 for irritation (Table 2.3).
- Eye irritation and serious eye damage. Category 1 is for corrosive effects and Categories 2A and 2B for irritation (Table 2.3).
- Skin or respiratory sensitization. If evidence is available to allow classification of sensitization, both skin and respiratory sensitization are in Category 1, but note the new symbol for serious effects for respiratory sensitization (Table 2.4).
- Single or repeated dose target organ systemic toxicity (TOST). This uses similar criteria for both single and repeated exposures (Table 2.4).
- Genotoxicity and germ cell toxicity. As with the EC criteria, there are two categories (Table 2.5).
- Reproductive toxicity. Reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring (Table 2.5).
- Carcinogenicity. As with the EC criteria, there are two broad categories (Table 2.6).

2.1.4 Acute Toxicity

Acute toxicity refers to those adverse effects presented upon single dose of a substance, or multiple doses given within 24 hours via oral or dermal administration, or by inhalation exposure of 4 hours. Chemicals can be categorized to one of five toxicity categories based on acute toxicity by the oral, dermal or inhalation route according to the numeric criteria expressed as LD50 (oral and dermal) or LC50 (inhalation) (UN 2011).

Acute systemic toxicity testing is defined as the potential of a substance to produce human hazard by determining its systemic toxicity in a test system based on the median lethal dose (LD50) value which means 50 percent of the test animal will be killed upon exposure of a single dose of the test substance. In order to have systemic toxic effects, a toxic substance must be absorbed by the body and distributed by the circulation, subsequently exerts its toxic effects. The observed toxicity is a result of liver biotransformation to convert a substance to a new form or metabolites. Depending on the route of exposure, acute systemic toxicity is assessed following oral, dermal and inhalation exposure.

The development of harmonized test method as the basis of any risk assessment procedure was established by the OECD's Environment, Health and Safety (EHS). Being considered as the leading international standard for safety testing and the development of new Test Guidelines (TG), the OECD Test Guidelines plays a vital role on testing and assessment. Together with GLP Principles, OECD Test Guidelines form an integrated part of the Council Decision on the Mutual Acceptance of Data (MAD) (Barlow *et al.* 2002).

Table 2.2 UN-GHS criteria, Single dose toxicity

	Toxic Category				
	1	2	3	4	5
					
Oral (mg/kg)	5	50	300	2000	Oral LD50 between 2000 and 5000 mg/kg. Indication of significant effect in humans. Any mortality in Category 4.
Dermal (mg/kg)	50	200	1000	2000	
Gases (ppm)	100	500	2500	5000	
Vapor (mg/L)	0.5	2	10	20	
Dust and mists (mg/L)	0.05	0.5	1	5	

(UN 2011)

Table 2.3 UN-GHS criteria, skin corrosion/irritation and serious eye damage/eye irritation

Skin corrosion/irritation		
Destruction of skin tissue; visible necrosis in one or more of three animals	Reversible adverse effects in skin tissue	
Category 1	Category 2	Category 3
		No pictogram
Subcategory 1A Exposure: 3 min or less Observation: Up to 60 min Subcategory 1B Exposure: 3 min and 1 hour Observation: Up to 14 days Subcategory 1C Exposure: 1 to 4 hours Observation : Up to 14 days	Exposure: 3 min	Mean irritation score of 1.5–2.3 for erythema/eschar or for edema in at least two or three tested animals at 24, 48 and 72 hours.

Serious eye damage/eye irritation		
Category 1	Category 2	
	Subcategory 2A Reversible in 21 days	Subcategory 2B Reversible in 7 days
		
Adverse effects on conjunctiva, cornea, iris that have not reversed within the observation period (normally 21 days after exposure) in at least one animal, and/or In at least two of three tested animals, a positive response of corneal opacity with a mean score of 3 or above, and/or a mean score of more than 1.5 for iritis, at 24, 48 and 72 hours.	Reversible adverse effects on conjunctiva, cornea, iris. Mean irritation score in at least two of three tested animals of 1 or more for corneal opacity and or 1 or more for iritis, and/or mean scores of 2 or more for redness and/or 2 or more for conjunctival edema (chemosis).	

(UN 2011)

Table 2.4 UN-GHS criteria, respiratory or skin sensitization and single or repeated target organ systemic toxicity (TOST)

Respiratory or skin sensitization	
Respiratory Category 1	Skin Category 1
	
Evidence in humans of specific respiratory sensitivity and/or Results of respiratory sensitivity from animal studies.	Evidence in humans of sensitization by skin contact in a substantial number of persons, or Results of skin sensitivity from appropriate animal studies.
Single or repeated target organ systemic toxicity (TOST)	
Category 1 Significant toxicity in humans	Category 2 Presumed to be harmful to human health
	
Reliable, good quality human case studies or epidemiological studies. Presumed significant toxicity in humans. Animal studies with significant and/or severe toxic effects relevant to humans at a generally (as a guide) low exposures.	Animal studies with significant toxic effects relevant to humans at generally moderate (as a guide) exposure. Human evidence in exceptional cases.

(UN 2011)

Table 2.5 UN-GHS criteria, germ cell mutagenicity and reproductive and developmental effects

Germ cell mutagenicity	
Category 1	Category 2
 Subcategory 1A Known to produce heritable mutations in human germ cells. Positive evidence from human epidemiological studies. Subcategory 1A Should be regarded as if they produce heritable mutations in the germ cells of humans, Positive results in: Human germ cell tests, In vivo heritable germ cell tests in mammals, In vivo somatic mutagenicity tests, combined with some evidence of germ cell mutagenicity.	 May induce heritable mutations in human germ cells. Positive evidence from tests in mammals and somatic cell tests. In vivo somatic genotoxicity supported by in vitro mutagenicity.
Reproductive and developmental effects	
Category 1	Category 2
 Known or presumed to cause effects on human reproductive ability/capacity or on development. Subcategory 1A Known (based on human data) Subcategory 1B Presumed (based on animal data)	 Suspected to cause effects on human reproductive ability/capacity or on development. Additional category effects on lactation or effects via lactation.

(UN 2011)

Table 2.6 UN-GHS criteria, carcinogenicity

Carcinogenicity	
Known or presumed human carcinogen Category 1 Category 2	Suspected human carcinogen
	
Subcategory 1A	Subcategory 1B
Known human carcinogen based on human evidence. Presumed human carcinogen based on demonstrated animal carcinogenicity.	Limited evidence of human or animal carcinogenicity.

(UN 2011)

An extensive set of safety tests is required for registration of new industrial chemicals, pesticides, pharmaceutical industrial chemicals, pesticides, pharmaceuticals and feed additives. However, according to MAD decisions, if the data developed in one country is accordance to the OECD Test Guidelines and GLP Principles, they are accepted for assessment purposes in all OECD countries. Hence, they may save a lot by avoiding repetitive testing and minimizing non-tariff barriers in trading products (Koe 2003).

As for the universal application, the United Nations has formulated a classification and labeling system which is called as 'Globally Harmonized System of Classification and Labeling of Chemicals' (UN-GHS) introduced in 2003 (UNECE 2003) with the most current revision published in 2011 (UNECE 2011). The UN-GHS classified four or five categories depending on geographical jurisdiction based on acute toxicity lethality values (LD50) of experimented animals in determining the acute systemic toxicity of a substance. Hence, LD50 values define the category boundaries in which becomes the European and UN-GHS frameworks of oral and dermal routes of exposure (Moore *et al.* 2013).

Harmonized criteria for health and environmental classification were developed for all of the various types of effects that were covered in the major existing systems. The intent is to continue this work in the OECD in the future, preferably by having national experts develop criteria for health or environmental effects of concern together so they are harmonized from the outset (Silk 2003).

Determination of acute dermal toxicity is important in evaluating and assessing the toxic characteristic of a substance when the route of exposure is by dermal route. As a short term exposure, acute dermal toxicity gives information on health hazards likely

to arise. In fact, the data collected from acute dermal toxicity provides a basis for classification and labeling. In addition, the data is also needed in determining the dose for sub chronic and other studies on dermal absorption (OECD 1987).

On the other hand, dermal toxicity testing is done to assess the local and/or systemic effects of a chemical after exposure to the dermal route. It only indicates either the penetration of a substance may produce a systemic toxicity not the amount of chemical absorbed (Basketter *et al.* 2012)

There are five Organization for Economic Cooperation and Development (OECD) Test Guidelines (TG) which describes acute systemic testing namely TG 402, TG 403, TG 420, TG423, and TG 425. The classification of chemicals is addressed by UN-GHS by hazard types and proposed harmonized hazard communication elements. This includes hazard classification criteria, labels, and safety data sheets. Besides, UN-GHS provides a cornerstone for harmonization of rules and regulations on chemicals at national, regional and worldwide levels (Sanderson & Thomsen 2009).

In order to determine the category of classification of GDMRC, acute dermal toxicity data is required. Thus, OECD TG 402 is chosen to review the hazard classification of this substance as it uses topical application on the skin.

2.2 Acute Dermal Toxicity Study

Acute dermal toxicity study is designed to evaluate and assess the systemic and localize toxicity effects of a substance when the route of exposure is via dermal route. As for OECD TG 402, acute dermal toxicity is defined as application of a test substance to no less than 10 percent of rats, rabbits, or guinea pigs skin followed by 14 days of observation. LD50 is determined by the number of animal's death and gross

pathological changes are to ascertain the relative toxicity of a substance (OECD, 1987).

In view dermal is a part of the human skin, the physiology and mechanism of skin absorption are explained in the next paragraphs.

2.2.1 Anatomy and Physiology of Skin

The skin is the largest organ of the body, with a total area of about 20 square feet and weighing about 5 kg with a surface area of 2 meter^2 (Godin & Touitou 2007). The skin protects us from microbes and the elements, helps regulate body temperature, and permits the sensations of touch, heat, and cold. This multi-layered organ acts mainly to protect the body from the surrounding environment, thus forming an efficient permeation barrier. There are two main layers of skin, i.e. epidermis and dermis.

The epidermis is the outermost layer of skin that provides a waterproof barrier and creates our skins color by special cells called melanocytes. The melanocytes produce the melanin pigment that gives a multi range of human skin colors. In epidermis layer, the major cell type is keratinocytes. The function of keratinocytes is to synthesize keratin, a protein linked by disulphide bonds to form supercoils polypeptide from coiled polypeptide chains (Venus *et al.* 2011). In this layer, it comprises of four compartments namely stratum basal, stratum spinosum, stratum granulosum and SC(SC) (Breitkreutz *et al.* 2013).

Stratum basal is the basal cell layer. It is only one cell thick and the main cell is keratinocyte. The melanocytes are also present and comprises of 5-10 percent of the cell population. Stratum spinosum is the prickle cell layer. As the basal cells move towards the surface it form a layer of polyhedral cells connected by desmosomes and seen as prickles under the microscope. Langerhans cells are present in stratum spinosum layer.

Stratum granulosum is the granular cell layer. Keratinocytes are found in the granular layer which contains intracellular granules of keratohyalin. The lipid contents are discharged from to cells into the intercellular space plays a vital role for the barrier function and intercellular cohesion within the SC. The barrier properties of the skin lie mainly within its outermost layer of the epidermis, SC. This highly hydrophobic layer is composed of differentiated non-nucleated cells, corneocytes (Venus *et al.* 2011), which are filled with keratins and embedded in the lipid domain (Godin & Touitou 2007).

The dermis is located beneath the epidermis, contains tough connective tissue, hair follicles, and sweat glands. In between the epidermis and dermal layer there is a basement membrane comprises of a thin layer of specialized extracellular matrix. As for the dermal layer which located under the basement layer, it consists of two regions which are papillary dermis and reticular dermis. In the papillary regions of the dermis it is composed of thin collagen fibers whereas the reticular dermis is composed of thicker, denser collagen fibers (Mikesh *et al.* 2013).

2.2.2 Mechanism of Skin Absorption

Skin absorption depends on a few factors that include contact area, duration of exposure, lipophilicity, test substance concentration, molecular weight, epidermis thickness and the integrity of SC (OECD 2004b). In addition, dermal penetration depends on the qualities of SC as it acts as an excellent barrier property by protecting the skin against penetration (Godin & Touitou 2007).

The dermal penetration studies are done in order to determine the amount of a chemical penetrate into the skin. This ensuring the substance has the potential to be absorbed into systemic circulation. Dermal penetration occurs by passive diffusion and

proceeds with the systemic absorption. However biotransformation of the chemical within the skin can happen before the systemic absorption (OECD 2004a). Previous study showed that the biotransformation at the human skin occurs due to the fact that it possesses multiple cytochrome P450 isoenzymes, influx and efflux transporter proteins. The cytochrome P450 isoenzymes located at the skin are different from the P450 isoenzymes in the liver but they can involve in both Phase I and Phase II metabolic reactions. Hence, the ultimate fate and bioavailability of a chemical may be influenced by dermal biotransformation (Abdallah *et al.* 2015).

In dermal absorption studies, human skin is preferably chosen for in vitro study whereas for in vivo studies, rat skin is used to observe the effect in human exposure. Rat skin represent for a worst case model for human skin. Even though rat skin is anatomically different in terms of thinner SC and more hair follicles as to compare to human skin, it is more permeable to chemicals and may represent for a worst case model for human skin (Aggarwal *et al.* 2015). Although the effect of dermal absorption is slower, prolonged exposure may cause severe toxicity. Thus, to enhance dermal absorption, lipophilic agent can be used and facilitated by the solvent and emulsifier used in the formulation (El- Naggar *et al.* 2009).

Molecules penetrate through SC via passive diffusion or via sweat glands and hair follicles directed towards dermis layer. The function of influx transport proteins in human skin in dermal uptake of xenobiotics is unknown. However, the role of Organic Anion Transporting Polypeptides (OATP) is highlighted in terms of mediating large organic cations via human keratinocytes by active transportation (Schi *et al.* 2003).

Other than the role played by SC as an epidermal barrier, other molecules such as xenobiotic metabolizing enzymes and transport protein act as a second biochemical barrier of the skin. Thus, chemical residues will be limited to the epidermis layer and eliminated from the exposed skin by desquamation so that it is not available for systemic distribution (Aggarwal *et al.* 2015).

Nevertheless, four mechanisms have been found to control the problem. In view SC has a tightly packed lipid region; skin resistance can be reduced by disrupting the permeability of lipid region. This causes increasing penetration through the intercellular lipid matrix. Another mechanism is by increasing the drug partitioning into the SC through formation of ion pair between the drug and fatty acid. Other possible mechanism is by increasing the drug solubility into the skin by having the drug to have high affinity for the solvent. Lastly is by increasing drug solubility in the vehicle such as acidic enhancers to increase the solubility of basic drugs (Gwak & Chun 2002).

2.2.3 Toxicity upon Dermal Exposure

Upon dermal exposure to hazard/chemical substance, two types of toxicity effects can be assessed namely localized dermal toxicity effects and systemic toxicity effects. Based on the UN-GHS health hazard groupings, localized dermal toxicity effects includes skin irritation/corrosion and skin sensitizing whereas systemic dermal toxicity effects includes acute dermal toxicity (Winder *et al.* 2005).

A localized dermal toxicity effect is an effect that is observed at the site of first contact, i.e. on the dermal. A systemic effect is an effect that is observed distant from the site of exposure, after the substance has become systemically available. In terms of localized dermal toxicity effects, there are a few criteria which may contribute

significantly to morbidity, i.e. edema, erythema/eschar formation, acanthosis, necrosis hyperkeratosis and papillomas, due to hazard exposure (Schaafsma *et al.* 2011).

The evaluation of systemic toxicity effects is due to percutaneous absorption of the test material whereas the local toxicity is determined by its contact with the skin. The absorption of the test material can be determined from observation of these effects either the material is sufficient to produce systemic effects or lethality (Arteaga *et al.* 2014)

Penetration through the skin and the toxic potency are the two factors that responsible for systemic toxicity of a chemical exposure. Skin penetration is different between substances as it is depending on lipid and water solubility of chemical through the skin. The toxicity effect cannot be determined by the amount of chemical on the skin surface. Thus, internal dose can be predicted by using experimental measurement of permeability coefficient (K_p) and steady-state flux. The other factor is by determining the toxic potency of a chemical by its mechanism of action. In measuring general toxicity, lethal dose (LD50) of half experimental animals, the lowest observable effect level (LOEL) and the no observable effect level (NOEL) are taken into account. Thus, it is possible if a nontoxic chemical has high rate of skin penetration compared to toxic chemical which do not penetrate skin at all (McDougal *et al.* 2007).

The world of skin cosmetics involves transdermal transportation of topically dermal applied substance. Other than penetrating into human skin via passive diffusion, topical formulation also enhance skin permeability to other bioactive ingredients. As a result, their percutaneous release can be evaluated and may become highly relevant for their quality and safety assessment (Gabbanini *et al.* 2009). Thus, the study focuses on acute dermal toxicity by employing OECD TG 402 method to elucidate systemic

effects. In addition, the rat was selected for this study as it may serve for the worst case scenario of human skin absorption study.

2.3 Insect Repellent

As part of personal protective measures, human contact with vector and nuisance arthropods will be reduced with the topical application of insect repellents. Repellents are used as primary importance against arthropod vectors when other methods are not accessible or impractical (Antwi *et al.* 2008).

2.3.1 Formulation

In view that an infected arthropod biting may result in disease transmission, the effectiveness of repellent products should be acknowledged to provide a prolonged protection from insect bites. Insect repellents can be classified into two types which are synthetic insect repellents and plant derived essential oil insect repellent (Fradin & Day 2002)**.** DEET is considered as a gold standard of insect repellent as it is developed and used since World War II by the US military. It is the most effective insect repellent which not only can be applied on skin, but also on fabrics. Natural insect repellent includes citronella oil, cedar oil, lemongrass oil and some others are listed by Environmental Protection Agency (EPA) to have minimum risk pesticides which enable them to be exempted from federal regulation (Katz *et al.* 2008; Leal 2014).

There are many types of residential insect repellent products including aerosol, mosquito coils, vaporizing mats, and liquid vaporizers which are easily accessible in the market (Sinha *et al.* 2006). These commercially available products are claimed to have repellency properties in repelling mosquito by the usage of botanical and natural remedy such citronella, eucalyptus, lemongrass and geraniol oil which have been

formulated into commercial repellents. However, protection time against bites may differ due to the volatility of botanical repellents thus it needs to reapplied time by time (Revay *et al.* 2013).

2.3.2 Mode of Action

Insect repellent generally is a chemical volatile substance which acts locally at a distance by preventing an arthropod from flying to, landing on or biting human or animal skin (Choochote *et al.* 2007). In order to maintain effectiveness of repellency properties, repellent must evaporate at vapor concentration at the skin surface so that repellent active agent may interact with an insect's olfactory system. Thus, the insect might repel from the host or confuse it so that it does not recognize the host (Karr *et al.* 2012).

Insect repellent may work in two ways which are either as odorant or as tastant. Working as an odorant, it provides a vapor barrier deterring the arthropod from coming into contact with the skin. The vapor produced making an offensive odor and bad taste and will repel the insects. The efficacy of the vapor depends on the boiling point of the chemical. Low boiling point chemicals will vaporize rapidly and degrade the product. Whereas high boiling point will create a desired repellent environment as it does not vaporize sufficiently. Thus, an optimum boiling point is between 230°F and 260°F to work effectively as insect repellent (Katz *et al.* 2008). As for nonvolatile compound, it cannot work as odorant which acts at a distance and disoriented the movement of the insect away from source. However, it can become a tastant by disengaging a mosquito from feeding (Leal 2014). Generally, the resemblance of the human skin from detection by insects and arthropods such as mosquito, gnats and ticks are the main function of insect repellent (Bissinger & Roe 2010).