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

IZZATI ABD MALIK

UNIVERSITI SAINS MALAYSIA

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by

IZZATI ABD MALIK

Thesis submitted in partial fulfilment of the requirements

for the degree of

Master of Science

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DECLARATION

I hereby declare that I am the sole author of this thesis in title "Hazard Classification Of Gamat- *N*,*N*-Diethyl-M-Toluamide (DEET) Mosquito Repellent Rubbing Oil 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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IZZATI ABD MALIK

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LIST OF SYMBOLS AND ABBREVIATIONS

DEET	N, N-diethyl-toluamide
GD MMRO	Gamat DEET Mosquito Repellent Rubbing Oil
GRO	Gamat Rubbing Oil
OECD	Organization for Economic Cooperation and Development
CPL	Classification on Packaging and Labelling
GHS	Globally Harmonized System
UN-GHS	United Nation of Globally Harmonized System
UN	United Nations
MW	Molecular Weight
EPA	Environmental Protection Agency
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
WHO	World Health Organization
GLP	Good Lab Practice
OATP	Organic Anion Transporting Polypeptides
K_p	Permeability coefficient
LOEL	Lowest Observable Effect Level
NOEL	No Observable Effect Level
RTBSA	Rats Total Body Surface Area
g	gram
ml	mililiter
mg/kg bw	milligram per kilogram body weight

ABSTRACT

The study is aimed to identify clinical signs exhibited by rats upon single exposure of Gamat-DEET Mosquito Repellent Rubbing Oil (GD MRRO) containing 15% of *N, N* Diethyl-m-toluamide as the active ingredient with bioactivity (repellent) against mosquitoes which includes Gamat Rubbing Oil (GRO) the placebo item. The study is performed to determine the hazard category of acute dermal toxicity of the GD MRRO 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 GD MRRO or the GRO, 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 GD MRRO satisfy the hazard toxicity class of Category U based on WHO Recommended Classification of Pesticides by Hazard (WHO, 2010) and the Class 5 of Acute Toxicity Hazard Categories under the United Nations Globally Harmonized System for Classification of Chemical Substances and Mixtures (UN-GHS).Therefore, the GD MRRO and the GRO are unlikely to present acute hazard upon dermal contact under normal use.

ABSTRAK

Kajian ini bertujuan untuk mengenal pasti tanda-tanda klinikal dipamerkan oleh tikus apabila terdedah kepada Minyak Sapu Penghalau Serangga Gamat dan DEET (GD MRRO) yang mengandungi 15% daripada *N, N* diethyl-m-diethyl sebagai bahan aktif dengan bioaktiviti (penghalau) terhadap nyamuk yang termasuk Minyak sapu Gamat (GRO) item plasebo. Kajian ini dilakukan untuk menentukan kategori bahaya ketoksikan kulit akut yang GD MRRO seterusnya untuk mengklasifikasikan ia berdasarkan kepada WHO Kelas Racun Bahaya dan UNGHS. 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 aplikasi 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 MRRO DEET atau GRO sehingga hari-14 terselamat tanpa kematian, hampir menemui ajal, sakit teruk atau kesusahan yang teruk. Tiada tanda-tanda klinikal yang berterusan atau tidak boleh diterbalikkan, diperhatikan di antara hari-1 sehingga kajian hari-14. Kesimpulannya, GD MRRO memenuhi kelas ketoksikan bahaya Kategori U berdasarkan WHO lawatan Pengkelasan Racun oleh Bahaya (WHO, 2010) dan Kelas 5 Kategori Ketoksikan Akut Bahaya di bawah UN-GHS .Therefore, yang GD MRRO dan GRO tidak mungkin untuk membentangkan bahaya akut apabila bersentuhan kulit di bawah penggunaan biasa

CHAPTER 1

INTRODUCTION

Mosquito borne diseases are disease affected by mosquitos which are responsible in transmitting diseases such as Yellow fever, dengue fever, malaria, Japanese Encephalitis and filariasis. Dengue fever is the most common life threatening viral infection causing millions of death every year with no proper vaccine or treatment (Sritabutra & Soonwera, 2013)

In Malaysia, there are 46681 cases has been reported on the incidence of dengue in 2014. This means that there is an increase of 246% cases compared to the year before (The Star Online, 2014). The figure indicated that this kind of mosquito borne disease is currently representing a greater health problem not only in Malaysia but several parts of the world. In order to avoid mosquitoes from biting the human, protection against them should be taken care of such as wearing protective clothing and application of repellent to the skin as the common approach.(Peter & Mwangi, 2014).

There are two types of commercial insect repellents which are synthetic chemicals and plant derived essential oils (Fradin and Day, 2002). A synthetic mosquito repellent which is widely used all over the world for past 60 years is *N*,*N*-diethyl-m-toluamide (Isman & Grieneisen, 2014). *N*,*N*-diethyl-meta-toluamide (DEET) is a wide-spectrum and most effective synthetic repellent available in the market due to its longer protection time and inexpensive. In terms of health concern, insect repellent which

contains DEET does not poses a health concern and is safe for the uses includes children (EPA, 2015). Commercial DEET formulations are available in several forms such as pressurized aerosol, aerosol pump spray, lotions and creams, liquids, roll-ons, and towellettes. (Costanza *et al.,* 2007).

In Malay communities, sea cucumber is regarded as one of traditional ointment product use in medicine industry and the locals named it as "Minyak Gamat" (Woo *et al.,* 2013). Gamat is actually one species of sea cucumber which is well known for its wound healing activity (Choo, 2004). In the present study, the finished product Gamat-DEET Mosquito Repellent Rubbing Oil (GD MRRO) is tested on acute dermal toxicity of *Sprague Dawley* rats in order to determine the hazard category of this product.

Gamat-DEET Mosquito Repellent Rubbing Oil (GD MRRO) is regarded as insecticide product. In view of GD MRRO that via topical application may cause direct exposure to human, the Malaysia Pesticide Board has mandated for any pesticide products to be evaluated for toxicological studies and provide safety data (*PESTICIDE ACT*, 1974)

In Malaysia Pesticide Board regulations also stated that, any manufactured pesticide substances to be registered for sale or distribution in Malaysia shall furnish non-clinical safety data. (P*ESTICIDE ACT*, 1974). Among the requirements in terms of toxicological data needed to register the product is acute dermal studies in rats. This is accordance to WHO Recommended Classification of Pesticides by Hazard Categories under the United Nation Globally Harmonized System for Classification of Chemical Substance and Mixtures (UN-GHS)

Table 1.1 Hazard class of an insecticide product is based on WHO Recommended Classification of Pesticides by Hazard (WHO, 2010) that has been aligned with the Acute Toxicity Hazard Categories under the United Nations Globally Harmonised System for Classification of Chemical Substances and Mixtures (UN-GHS)

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

In view of this, this study is done to better evaluate of the oil formulation with DEET and without DEET which offer high repellency as well as good consumer safety. Thus, this study aims to determine the hazard category of Gamat-DEET Mosquito Repellent Rubbing Oil based on the UN-GHS classification with three main objectives which are;

1. To identify clinical signs exhibited by the rats upon single dosing of the test item (Gamat-DEET Mosquito Repellent Rubbing Oil) and the placebo (Gamat Rubbing Oil), subsequently determining the need for repeated dose toxicity study based on the outcome of necropsy gross examination.

2. To determine the hazard category of the test item (Gamat-DEET Mosquito Repellent Rubbing Oil) 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 Class 4 and above.

3. To determine the hazard category of the placebo item (Gamat Rubbing Oil) to elucidate the possible toxicity contributed by the inert ingredients.

In conclusion, this study is important in determining the hazard class category of the product in order to safeguard the consumer in terms of its health hazard that the product may produce. It is also will help local business entrepreneur to market their product in European Country in the future.

CHAPTER 2

LITERATURE REVIEW

2.1 United Nation of Globally Harmonized System (UN-GHS)

As a worldwide practice, chemicals substance are used to enhance and improve life especially in consumers‟ products. However, these chemical substances have the potential to produce adverse effect (UNECE, 2011). In most countries, the transmission of information about the hazard of chemical substances are developed through labels and/or safety data sheets. Nonetheless, due to inconsistencies in countries regarding classification and labeling of a same chemical substances, it may give a strong impact on both the protection to human health and environment (Winder *et al.,* 2005). Thus, a proper classification and labelling regarding the use of the chemicals substance may draw attention of the user about the hazards and protect them from the hazards of these chemicals. Since there are diverse systems of classification and labelling of chemical substance around the world, there is a need for an internationally harmonized approach to be regulated in order to standardize the systems (UNECE, 2011).

The presence of multiple existing laws or regulations on chemical classification and labelling such as United Nation (UN) Dangerous Goods System, European Community (EC) for system for hazardous substances (Winder *et al.,* 2005) and in Malaysia, CPL Regulation 1997 (OSHA, 1994) have variations in definitions of hazards and caused confusion at user level.

As a result, at the Rio Summit in 1992, Agenda 21 has developed the concept of Globally Harmonized System (GHS) where it recommended the implementation

of GHS for classification and labelling of chemical substances. In 2003, United Nations (UN) collaborated with Organization for Economic Cooperation and Development (OECD) introduced „Globally Harmonized System of Classification and Labelling of Chemicals" (GHS) (UNECE, 2003) with the most current revision published in 2011 (UNECE, 2011). Thus, UN-GHS chemical classification is purposely developed to standardize worldwide safe chemical management issues from manufacturer, transport, usage and disposal (Winder *et al.,* 2005).

2.1.1 Hazard Classification

As for the term of hazard classification, it includes the compilation of all relevant data need to be carried out by the supplier to place the chemical substance or products on the European Union market. In order to determine the hazard class, a cut off values for maximum levels is to be considered when evaluating test result of animal experiments. These cut-off values shall reflect a threshold between toxicological relevance and non-relevance to humans covering all exposure situations presumably appearing under real-life conditions. Thus, a proper classification and labelling is required to identify and specify the hazard class of the chemical substance or the product (Gebel *et al.,* 2009).

As an internationally agreed system for classification and labelling of chemical substances or mixtures, GHS classified them in terms of physical hazard, health (toxicology) hazard and environmental hazard which covers range of consumer products, workplace, pesticides and transportation. Implementation of GHS not only protect human health and environment but indirectly promote chemical trade between countries all over the world (Thannimalay & Yusoff, 2008). Below are the three categories

a) Physical Hazard : Chemical substances in this category will be classified not only for hazard type (hazard class, e.g flammability) but also the degree of hazard presented (hazard category e.g extremely flammable, highly flammable).

b) Toxicology (or Health) Hazard : In this category, a chemical substance is concerned about its potential to posed health effects to human.

c) Ecotoxicological (or Environmental) Hazard : In environmental hazard, it classified chemical substances which are hazardous for the aquatic environment. (Winder *et al.,* 2005)

The communication of hazard in the GHS is based on the provision of signal words, hazard statements and pictograms, all of which are linked to the specific hazard of the substance or mixture (Silk, 2003).

In European market, chemical substances are regulated by the European Union Regulation on chemicals and their safe use commonly known as Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) in 2007 (Gebel *et al.,* 2009). REACH represents a major piece of chemical legislation in Europe which aims to ensure a high level of protection of humans and the environment by classifying them according to their physicochemical, environmental or human health hazards (Oltmanns *et al.,* 2014).

2.1.2 Health Hazard

The term health hazard can be defined as chemical substance which may result in acute or chronic health effects to a person based on at least one study conducted with established scientific principles. The health hazard that may occur would be in terms of carcinogens, toxic or highly toxic agents, reproductive, irritants,

corrosives, sensitizers, hepatotoxins, nephrotoxins, neurotoxins and agent which may cause damage to heamatopoietic system and respiratory system (ILIP, 2015).

In health hazard classification, it 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 is a summary of all criteria have been developed for the classification of the following types of health effects according to GHS classification system :

a) Single dose toxicity, covering a range of toxicity endpoints by various routes of exposures (Table 2.1).

b) Skin irritation and corrosion. As for Category 1 is for corrosive effects and Categories 2 and 3 for irritation (Table 2.2).

c) Eye irritation and serious eye damage. As for Category 1 is for corrosive effects and Categories 2A and 2B for irritation (Table 2.2)

d) Skin or respiratory sensitisation. As for serious effect of sensitization, new label is shown in Table 2.4.

e) Single or repeated dose target organ systemic toxicity (TOST). This uses similar criteria for both single and repeated exposures (Table 2.4).

f) Genotoxicity and germ cell toxicity as shown in Table 2.5.

g) Reproductive toxicity as well as developmental toxicity in the offspring (Table 2.5).

f) Carcinogenicity criteria with two broad categories (Table 2.6).

(Winder *et al.,* 2005)

Table 2.1 GHS Criteria, single dose toxicity

	Toxicity 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 Indication from other studies
Dermal (mg/kg)	50	200	1000	2000	
Gases (ppm)	100	500	2500	5000	
Vapour (mg/L)	0.5	2	10	20	
Dust and mists (mg/L)	0.05	0.5	1	5	

(Winder *et al.*, 2005)

Table 2.2 GHS criteria, skin corrosion/irritation and serious eye damage/eye 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
Subcategory 1A	Subcategory 1B	Subcategory 1C		
Exposure: 3 min or less Observation: Up to 60 min	Exposure: 3 min and 1h Observation: Up to 14 days	Exposure: between 1 and 4h Observation : Up to 14 days	Exposure: 3 min or	Mean irritation score of 1.5–2.3 for erythema/eschar or for oedema in at least two of three tested animals at 24, 48 and 72h

Category 1 	Category 2	
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 h	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 oedema (chemosis)	
	Subcategory 2A 	Subcategory 2B
	Reversible in 21 days	Reversible in 7 days

(Winder et al., 2005)

Table 2.3 GHS criteria, respiratory or skin sensitization and single or repeated target organ systemic toxicity (TOST)

Respiratory sensitization	Category 1
	 Evidence in humans of specific respiratory sensitivity and/or Results of respiratory sensitivity from animal studies
Skin sensitization	Category 1
	 Evidence in humans of sensitisation by skin contact in a substantial number of persons, or Results of skin sensitivity from appropriate animal studies

Category 1	Category 2
	
Significant toxicity in humans Significant toxicity in humans 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	Presumed to be harmful to human health Animal studies with significant toxic effects relevant to humans at generally moderate (as a guide) exposure Human evidence in exceptional cases

(Winder *et al.*, 2005)

Table 2.4 GHS criteria, germ cell mutagenicity and reproductive and developmental effects

Category 1		Category 2
		
Subcategory 1A	Subcategory 1A	May induce heritable mutations in human germ cells
Known to produce heritable mutations in human germ cells Positive evidence from human epidemiological studies	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	Positive evidence from tests in mammals and somatic cell tests In vivo somatic genotoxicity supported by in vitro mutagenicity

Category 1	Category 2
	
Known or presumed to cause effects on human reproductive ability/capacity or on development	Suspected to cause effects on human reproductive ability/capacity or on development

Subcategory 1A	Subcategory 1B	Additional category effects on lactation Or effects via lactation
Known (based on human data) Presumed	Presumed (based on animal data) Suspected	

(Winder *et al.*, 2005)

Table 2.5 GHS criteria, carcinogenicity

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

(Winder *et al.*, 2005)

2.1.3 Acute Toxicity

In marketing chemicals for international distribution, the estimation of mammalian acute systemic toxicity is important. Concerning on the regulatory respond for hazard classification and labelling by industrial chemicals, agrochemicals, biocides and pharmaceuticals, acute toxicity is conducted. (Seidle *et al.,* 2010). Thus, GHS has implemented rules for classification of human acute toxicity categories based on experimental findings (UN, 2011). The findings are obtained from oral, dermal or inhalation of single dose exposure of rodents and calculation lethality based on concentration effect (Seidle *et al.,* 2010).

There are five acute toxicity categories to determine certain threshold for LD_{50} namely : category I to III is labelled as „danger", category IV is labelled as „warning" and category V has no label. As for category V, compounds are comprised of low acute systemic toxicity. Different categories has certain threshold which are specific for the exposure route (i.e oral, dermal, or inhalation). The „warning" label is to classify the most severe hazard category (UN, 2011).

According to GHS, acute toxicity is defined as "those adverse effects occurring following oral or dermal administration of a single dose of a substance, or multiple doses given within 24 hours, or an inhalation exposure of 4 hours". In another word, acute toxicity is the potential of a substance to produce human hazard by determining its systemic toxicity in a test system based on the median lethal dose (LD_{50}) value which means 50% of the test animal will kill upon exposure of a single dose of test substance (UNECE, 2004).

Acute toxicity testing also is called as acute systemic toxicity. In order to have systemic toxic effects, a toxic substance must be absorbed by the body. Then, the toxic substance is distributed by the circulation and exerts its toxic effects to the target cell or organ. The observed toxicity is a result of liver biotransformation to convert a drug or chemical to a new form or metabolites. There are five (OECD) Test Guidelines (TG) which describes acute systemic testing namely TG 402, TG 403, TG 420, TG423, and TG 425. As for the testing guidelines, World Health Organization (WHO) through GHS (UN, 2011) has recommended testing procedures by referring to Organization for Economic Cooperation and Development (OECD) testing guidelines (Scholz *et al.,* 2013).

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. Due to this respect, OECD‟s Environment, Health and Safety (EHS) has established the development of harmonized test method as the basis of any risk assessment procedure (Barlow *et al.,* 2002).

As for new chemical substances of products, the active compound and final commercial products are required to be proven safe before entering European market. (Scholz *et al.,* 2013). Thus, any data developed in one country accordance to Good Laboratory Principle (GLP) and OECD Test Guidelines, they are accepted for assessment purposes in all OECD countries. As a result, it may save a lot by avoiding duplicative testing and minimizing non-tariff barriers in trading products (Koe, 2003).

2.2 Acute Dermal Toxicity Study

Acute dermal toxicity is defined as application of a test substance to no less than 10% of rats, rabbits, or guinea pigs skin followed by 14 days of observation. LD_{50} is determined by the number of animals‟ death and gross pathological changes are to ascertain the relative toxicity of a substance. Acute dermal toxicity study is referred as OECD Test Guidelines 402 (OECD, 1987).

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 labelling. In addition, the data also needed in determining the dose for subchronic and other studies on dermal absorption (OECD, 1987).

2.2.1 Skin anatomy and physiology

Skin is regarded as the largest organ in human body weighing about 5 kg with a surface area of 2 m^2 (Godin & Touitou, 2007). Skin functions as protection from surrounding environment by forming an efficient permeation barrier for exogenous molecules. 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 namely epidermis and dermis as shown in Figure 2.1.

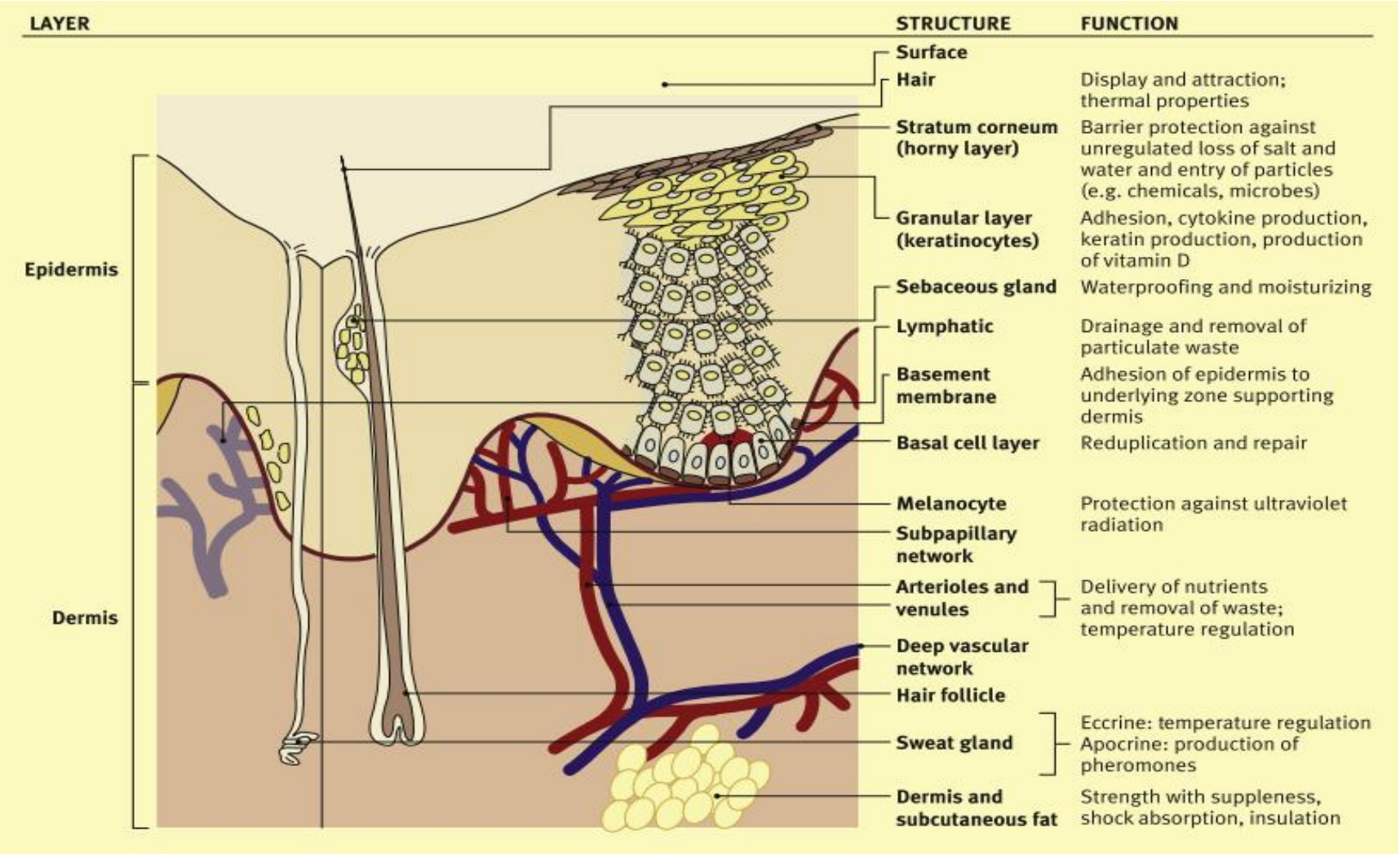

Figure 2.**1** Skin structure and its function (Venus *et al.*, 2011)

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).

Epidermis represents the outer layer of organism which acts as a protection barrier to penetration of chemicals to the underlying vascular dermis (Venus *et al*., 2011). It comprises of four compartments namely stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Breitkreutz *et al*, 2013). Below are the description of each stratum :

- Stratum basale (basal cell layer)

  In this layer, it is only one cell thick and the main cell is keratinocyte. The melanocyte is also present and comprises of 5-10% of the cell population.

- Stratum spinosum (spinous or 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 this layer.

- Stratum granulosum (Granular cell layer)

  In this layer, keratinocytes are found in the granular layer which contains intracellular granules of keratohyalin. The lipid contents is discharged from to

cells into the intercellular space plays a vital role for the barrier function and intercellular cohesion within the stratum corneum.

- Stratum corneum (horny layer)

  The outermost layer of the epidermis is stratum corneum in which the cells migrated from stratum granulosum. The cells in this layer are called as corneocytes with nuclei and cytoplasmic organelles have been lost (Venus *et al.*, 2011).

2.2.2 Mechanism of Skin Absorption

In order to determine the amount of a chemical penetrate into the skin, dermal penetration study is done. It is done to determine the potential of the substance to be absorbed into systemic circulation (OECD, 2004a). However, before systemic absorption occurs, biotransformation of the test substance within the skin can take place before dermal penetration by passive diffusion.

In latest study shows that biotransformation of human skin occur due to the fact that it possess multiple cytochrome P450 isoenzymes, influx and efflux transporter proteins. Although the patterns of cytochrome P450 isoenzymes differ from liver, it seems that skin can involve in both Phase I and Phase II metabolic reactions. There are at least five different esterases reported to work upon simple ester bonds such as in organophosphate compounds. Therefore, the ultimate fate and bioavailability of a chemical may be influenced by dermal biotransformation (Abdallah *et al.,* 2015)

Skin absorption may occur depending on a few factors that affect the permeation of a substance through the skin. This includes contact area, duration of exposure,

lipophilicity, test substance concentration, molecular weight, epidermis thickness and the integrity of stratum corneum (OECD, 2004b). The rate if dermal penetration depends on the qualities of stratum corneum as it acts as excellent barrier property by protecting the skin against penetration.

In dermal absorption studies, human skin is preferably chosen for in vitro study whereas for in vivo studies, rat skin is used to see 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 stratum corneum and more hair follicles as to compare to human skin, it is more permeable to chemicals (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 helped by the solvent and emulsifier used in the formulation (Naggar *et al.,* 2009).

Stratum corneum (SC) provides the barrier properties of the skin and contains up to 16 cell layers and it takes up two weeks to completely desquamate (Hoath & Leahy, 2006). SC has highly hydrophobic layer which composed of differentiated non-nucleated cells, corneocytes filled with keratins, and embedded in the lipid domain. 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 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 stratum corneum as epidermal barrier, other molecules such as xenobiotic metabolizing enzymes and transport protein act as 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).

However, there are four mechanism has been found to overcome to this problem. Since stratum corneum has a tightly packed lipid region, skin resistance can be reduced by disrupting the permeability of lipid region which result in increasing penetration through the intercellular lipid matrix. Other possible mechanism is by increasing the drug partitioning into the stratum corneum through formation of ion pair between the drug and fatty acid. Another 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 effect upon skin exposure

Acute dermal toxicity testing is designed to assess the local and/or systemic effects of a chemical after exposed to the dermal route. It only indicates either the penetration of a substance may produce a systemic toxicity not the amount of chemical absorbed. 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).

Local effects due to dermal exposure can be described into a few criteria such as, oedema, eschar formation, acanthosis, necrosis hyperkeratosis and papillomas. All the criteria may contribute significantly to morbidity (Schaafsma *et al.*,, 2011). Skin erythema can be defined as an inflammatory disorder that produces tender red bump (nodules) under the skin. The factor that might cause erythema is due to sensitivity of a drug. Edema is an allergic reaction caused by sensitivity of certain irritants or chemical substances. Edema is reversible as it may return to normal once the cause is identified and eliminated whereas eschar is termed as dead tissue cell due to skin injury (Arteaga et al., 2014)

In order to assess systemic toxicity of a chemical exposure, two factors are responsible for it which are penetration through the skin and the toxic potency. As skin is a good water barrier, skin penetration is different between substance 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).

In cosmetic formulation, essential oil such as in perfumes and massage oil are employed. By penetrating into human skin via passive diffusion, topical formulation also

enhance skin permeability to other bioactive ingredients. As a result, their percutaneous release is can be evaluated and may become highly relevant for their quality and safety assessment (Gabbanini *et al.*, 2009).

2.3 Insect Repellent

Insect repellent can be defined as a chemical volatile substance which act locally at a distance by preventing an arthropod from flying to, landing on or biting human or animal skin (Choochote *et al.,* 2007). An ideal repellent should have longer protection against biting insects and does not cause adverse effect (Lupi *et al.,* 2013). It can be classified into two types which are synthetic insect repellents and plant derived essential oil insect repellent (Fradin & Day, 2002)**.**

Synthetic insect repellent such as *N*,*N*-diethyl-m-toluamide (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).

2.3.1 Formulation

There are many formulations of insect repellent available in the market. This includes insect repellents in chemical forms such as sprays, creams, lotions, aerosols, oils and grease sticks (Lupi *et al.,* 2013). As for insecticidal products which are easily accessible in the market also include mosquito coils, vaporizing mats and liquid vaporizers (Sinha *et al.,* 2006). As part of personal protection measures, topical

application of insect repellents will reduce human contact with vector and nuisance arthropods. Therefore, when other methods are not accessible or impractical, topical repellents are used as primary importance against arthropod vectors (Antwi *et al.,* 2008).

The efficacy of a pesticidal products is depending on the type of formulations. External factors such as air temperature, humidity and wind speed may influence the repellency efficacy in warm and humid climates. As for example, in condition of warm and humid climates, the duration of effectiveness is generally slower. Thus, more reapplications of the repellents are needed. The repellency effectiveness is decreased by evaporation rate and percutaneous penetration. As for example, products which contained are able to penetrate deeper into skin which result in loss of effectiveness (Lupi *et al.,*. 2013).

The primary mechanism which limits the duration of efficacy due to loss of active ingredients are through percutaneous and evaporation, product wash off or rub off. Thus, to increase activity loss, ethanol was used in commercial formulation as primary ingredient of DEET permeation enhancer (Karr *et al.,* 2012).

According to the WHO Recommended Classification of Pesticides by Hazard in 2009, it stated that any products intended for final classification of a formulation should be based on toxicity data obtained on that formulation by the manufacturer (WHO, 2009).

In formulation of final product of pesticide, active and inert ingredients are combined. By definition, an inert ingredient is an intentionally added substances to a pesticidal product. This is done to enhance the performance of the product such as