ACUTE ORAL TOXICITY CLASSIFICATION OF *Physalis minima* LEAVES CRUDE WATER EXTRACT USING THE OECD TG423 ON FEMALE SPRAGUE DAWLEY RATS

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By

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DECLARATION

I hereby declare that I am the solitary author of this thesis entitled **'Acute oral toxicity classification of *Physalis minima* leaves crude water extract using the OECD TG423 on female Sprague Dawley rats'**. I declare this thesis is being submitted to Universiti Sains Malaysia (USM) for the purpose of the award of Master Science in Health Toxicology. This dissertation is the result of my own research under supervision of Dr Hasni b. Arsad except for citations in the references which have been duty acknowledged. The dissertation has been accepted for the study performed and is not concurrently submitted in the candidature of any other institutions.

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ABSTRAK

Daun *P. minima* atau dikenali sebagai Daun Letup-letup, adalah tumbuhan yang biasanya digunakan atau diaplikasikan oleh masyarakat tempatan di Malaysia sebagai ubat-ubatan. *P. minima* juga menunjukkan kepelbagaian aktiviti biologikal dan farmakologikal di dalam beberapa kajian dimana tumbuhan ini menunjukkan pengesahan di dalam penggunaan terapeutik. Tumbuhan ini dipercayai mempunyai ciri-ciri medikal untuk penyakit saraf, sawan, antioksida hati, anti-inflamasi, melegakan kesakitan, mengurangkan demam dan membantu penyembuhan masalah pundi kencing. Ketoksikan pokok *P. minima* sangat penting dalam mengkelaskan tahap keselamatannya. Eksperimen ini berjalan mengikut garis panduan OECD 423 dimana 2000 mg/kg takat dos dijalankan keatas tikus betina Sprague Dawley (210-260g) dan selama 14 hari tikus diperhatikan sepanjang eksperiment berjalan. Sebanyak enam ekor tikus digunakan untuk uji kaji ini. Berat badan setiap tikus direkodkan pada hari ke-7 dan ke-14. Kemudian pada hari ke-14, kesemuanya dieutenasi dengan karbon dioksida dan beberapa organ dipilih (otak, buah pinggang, hati, jantung, limpa), ditimbang dan organ-organ tersebut diteruskan bagi menganalisa histopatologi tikus. Berdasarkan logic keputusan UN-GHS, oral akut 2000 mg/kg *P. minima* diklasifikasikan dibawah kategori tidak diklasifikasikan.

ACUTE ORAL TOXICITY CLASSIFICATION OF *Physalis minima* LEAVES CRUDE WATER EXTRACT USING THE OECD TG423 ON FEMALE SPRAGUE DAWLEY RATS

ABSTRACT

The leaves of *Physalis minima* commonly known as "Daun Letup-letup" is a plant which is commonly used by the local community in Malaysia as a natural remedy in folk medicine and possess several bioactivities. *P. minima* exhibited a wide range of biological and pharmacological activities in a few laboratories studies and believed to have system of medicines for nervous disorder, epilepsy, liver antioxidant, anti-inflammation, analgesic, antipyretic and as a helpful remedy in ulceration of the bladder. The toxicity of *P. minima* was important to identify of its safety. In order to assess the toxic class category of *P. minima*, OECD TG 423 was applied to determine *P. minima* toxicity involving limit dose of 2000 mg/kg by using healthy female Sprague Dawley albino rats (210-260 kg) were closely observed for 14 days. Six animals were used for each test. Acute toxicity study of *P. minima* extract was suspended in water and administered by oral gavage**.** Bodyweight for each rats was rrecorded at day 7 and day 14. All treated female SD rats survived the 14 day observation period and surviving animals were euthanized with carbon dioxide at day 14 and various tissues (brain, kidneys, liver, lung and spleen) collected, weighed and visually inspected for gross necropsy and histopathology was performed for further histopathological analysis. Based on UN-GHS decision logic, acute exposure of 2000 mg/kg of *P. minima* was safe in female rats without causing any adverse effects or mortality and was clasified under unclassified category.

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LIST OF ABBREVIATIONS

°C	Degree Celcius
ANOVA	Analysis of Variance
g	Gram
GB	Gigabite
kg	Kilogram
mg	Milligram
OECD	Organization for Economic Co-operation and Development
P. minima	Physalis minima
SPSS	Statistical Package for the Social Science
UN-GHS	United Nation-Globally Harmonized System
Bw	Body weight

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Interaction between medicinal plant with the health care system seem like having the probability to provide a compound of complex and unique structure which have capability used by mankind as therapeutic (Khan *et al*., 2009). Aqil (2006) stated that about 60 to 80% world population still depends on plant based medicines which are being used since the ancient ages as the traditional biological system and the largest source of herbal medicine is made up from world plant biodiversity. *Physalis minima,* belonged to Solanacea is an annual herb having 0.5 to 1.5 m height and it is known as native gooseberry, country gooseberry, wild Cape gooseberry. This plant widely found throughout India, Baluchistan, Afghanistan, Tropical Africa, Singapore, Malaysia and Australia. The fruit is encapsulated in papery cover, yellowish in color and local community commonly eats this fruit (Plate 1.1). In review literature Parmar and Kaushal (1982) approved that this kind of plant's fruit exhibit good foundation of vitamin C and was considered to be a purgative, diuretic and used to cure spleen disorder and relieve pain (analgesic action). Ethnobotanical information showed that *P. minima* has tremendous medicinal value for the cure out different diseases (Arvind *et al*., 2011).

Plate 1.1: *Physalis minima* with leaves are soft and smooth (not furry), cream to yellowish flowers are followed by edible yellowish fruit encapsulated in papery cover.

Very slightly is known about *P. minima* among community, especially regarding its phytochemistry, safety, pharmacological and biological activities in the worldwide (James and Peter, 2010). Till date, utmost of the previous study have been reviewed regarding this plant, however, no proof or confirmation of experimental on its toxicity even though the fact is this plant have been studied of their several pharmacological activity. So far, no study has been done on the toxicity of *P. minima* leaves water extract. The intention is to accomplish evidence on *P. minima* safety and offer guidance for selecting a safety dose of *P. minima* in its usage in traditional medicine. The traditional decoction dosage form of *P. minima* could be imitated by using the water extract. Hence, purpose of this study is to investigate the histopathological toxicity of *P. minima* water extract by acute oral administration in female rats based on OECD 423 guideline.

1.2 Objectives of the Study

The objectives of this present study have been restricted to the following:

i) To determine the acute hazard category of *P. minima* leaves crude water extract in rats based on the OECD TG423.
ii) To investigate the toxicophatological effect of *P. minima* leaves crude water extract on Sprague Dawley rats organ.

CHAPTER 2

LITERATURE REVIEW

2.1 *Physalis minima*

2.1.1 Medical Importance

Physalis minima materials exhibited a wide-range of pharmacological and biological actions in a few laboratory studies, which may influence its validating for therapeutic use in traditional medicine. For instance, previous study reported this plant as one of the important medicinal plants in Indian traditional system of medicines (Daya and Vaghasiya, 2012) for nervous disorders, epilepsy, bronchitis, liver ailments and also used as an anticardiovascular (Jyothibasu *et al*., 2011). It also protects against hepatoxicity induced by CCl4 and as a liver antioxidant (Sreepriya *et al*., 2001). Anon (1969) reported that this plant exhibited tonic, diuretic and laxative properties. Additionally, even it is bitter, the plant *P. minima* acts as appetizer, ascites, spleen enlargement, applied in inflammations and by means of the worthiness remedy in bladder ulceration. Karthikeyani and Janardhanan (2003) approved the leaves are applied over snakebite site once it crushed. The bis-indoles family known generically as indirubins is the main constituents of *P. minima*, a product from Chinese material medical which is used to treat myelogenous leukemia and possess cytotoxic activity (Cragg *et al*., 2005). Ooi (2010) stated that *P. minima* has antiproliferative effect on several cancer cell lines.

Kirtikar and Basu (1980) agreed that decoction of leaves of this *P. minima* plant also used for horses and gonorrhoea. The fruit is considered alternative, diuretic, and aperients, being useful in dropsy, urinary disease, and gout. They reported that the fruit is said to infuse vigor in a worn-out system and to cure premature decay. Root is used as a vermifuge and used for fever. Decoction of root is used for diabetes. The fruit is used for gout. Poultice of fruits used for headaches and intestinal pain. Kurian (1990) found out that the other uses of *P. Minima* is laxative, expectorant or promoting the discharge of phlegm, painful discharge of urine, ulcer, cough and bronchitis.

Furthermore, many steroidal lactones have been identified from the plant and it has been reported to possess antifertility activity in Wistar rats (Sudhakaran *et al*., 1999), study on ethanolic extract is reported to possess in vitro inhibitory activity on intestinal alpha glucosidase maltase and hypoglycemic effect (Raju, 2010), the chloroform extract of *P. minima* is reported to possess cytotoxic actions on NCI-H23 cell line (Leong and Tengku Muhammad, 2010). Moreover, Gupta (2010) reported that this plant when tested by disc diffusion method against Gram positive and Gram negative microorganisms (bacteria), has shown anti-ulcer activity in rats, and possess antibacterial activity. Furthermore, the whole plant of the chloroform fraction and crude methanol extract has been reported to possess analgesic, anti-inflammatory and fever infection activities in Wistar rats and NMRI mice (Murad Ali Khan *et al*., 2009). Antimalarial (The Wealth of India, 2003), and anti-gonorrhoeal activity (Caceres, 1995) has been identified by previous researchers. There is a previous study Zakaria and Mohamad (2010) did mention that the whole decoction of this *P. Minima* plant is consumed as a medicine for cancer by the Malay community. Vimal Kumar (1990) has identified the plant *P. minima* exhibits strong anticancer, antiviral and several other

activities. As in several medicinal plants, these properties may be due to the presence of alkaloids, cardiac glycosides steroids, flavonoids, terpenoids and tannins in the crude extracts of *P. minima*. In Benggali, the plant *P. minima* Linn. local name is known as Tepari or Patka which may have potential use for leukemia chemotherapy. Leaves and fruits are purgative, tonic, diuretic and used in spleen disorders and gonorrhoea (Ahsan *et al*., 2009).

Hence, the pilot screening test may lead to benefits for screening of drug discovery and development and afterward in the detection of the bioactive principles. The clinical observation of rat and adverse effect of toxicity was observed continuously after the oral administration of plant extract for 1 hour, subsequently alternating for 4 hours, above a period of 24 hours and up to 14 days (Twaij *et al*., 1983).

2.1.2 Nutritional Importance of *Physalis minima* Plant

In a study done by Parmar (1982), since the fruit is juicy, it comprised 76.7% moisture and 61.4% of extractable juice. This fruit is rich and contain a good amount of vitamin C and the overall it comprises 12.5% of soluble solids content of the juice. The acidity of the juice is 1.81%. Likewise, it consists of 5.97% total sugars, 2.81% non-reducing sugars, 3% reducing sugars, 0.52% pectin and 0.64% tannins. The as represented by its residue, mineral content of the fruit is 1.23% and 2.75% representing the protein content of the fruit. The content of some of the significant minerals of the fruits consisting viz phosphorus (0.108%) magnesium (0.613%), potassium (0.024%), calcium (0.056%), and iron (0.006%) respectively.

2.2 UN-GHS

With veneration during Earth Summit 'Rio Declaration' in 1992, United Nation-Globally Harmonized system (UN-GHS) has been developed for worldwide solicitation. It is one way of sharing the knowledge of hazard communication (Jahangir, 2013). There are many benefits that industries and public can gain when implementing the GHS. Firstly, it provides enhanced health and environmental protection to people who are exposed to chemical through better precision and regulatory provided. Seguin (2009) stated that this system has been targeted to produce or be responsible for label messages consistency to flawless, emergency first responders, substance handler, consumers and the public.

Moreover, Seguin (2009) indicates that by implementing the GHS, barrier to trade and facilitate acquiescence can be minimized. This was done by eliminating dependence to comply with communication system and hazard classification. In addition, GHS provide users by safety as well, guaranteeing more consistent hazard communication, environmental protection and a good health (Seguin, 2009). Lastly, by ensuring an improved information flow up and down the chemical source chain, GHS will help supervisors, manufacturing and consumers (Seguin, 2009). The application of GHS in standardizing the hazard information and label will have a big impact on the chemical industry that manufacture and supplies chemical and also the use of chemical. In fulfilling the GHS compliance, the acute toxicity testing is one way of the method.

2.2.1 OECD Test Guidelines 420, 423 and 425

This Guidance Document intended to assist with the most appropriate choice of guideline to enable particular data required to be met while decreasing the use of animal suffering and animal number, hence providing material for both the regulated community and regulators. The Guidance Document comprises of Guidelines 420, 423 and 425 which contain additional information on the demeanour and clarification in this guideline.

Chan (1994) stated that the guideline entirely involves a fasted well young adult rodent with single dose administration of the test material by oral gavage, subsequently after dosing, the rats are under observation for up to 14 days, the body weight is noted down and then, all animals undergo necropsy for further analysis. A constant volume or a constant concentration of doses may be administered based on depending upon the requirements of the regulatory authorities and the toxicologist. On the day of dosing, each animal selected from the available animals randomly.

A finding study use Guideline 420 which acts in accordance with an appropriate starting dose and reduction number of animals used. Dose 5, 50, 300 or 2000 mg/kg are used both in the main and finding study of pre-specified fixed doses. In order to use an additional dose level of 5000 mg/kg, an option has been made, but only when justified by a precise regulatory need. Some sign of toxicity may probably produce with the initial dose being selected as the dose, groups of animals are dosed in a stepwise procedure. The next groups of animal may be at higher or lower fixed doses, in order to achieve the objective, if only the sign of toxicity is present. All above depiction of the test substance classification is based on the dose identification which may cause evident

toxicity, with the exception of when there are no effects at highest fixed dose (OECD, 2001).

Pre-specific fixed dose of 5, 50, 300 or 2000 mg/kg are used for Guideline 423. There is a selection to use an additional dose level of 5000 mg/kg, but only in condition when vindicated by specific regulatory requirement. Ensuring mortality in some animals, the initial dose administration is necessary with group of animal are dosed in a stepwise procedure. In order to achieve the study objective, practically animal may be dosed at higher or lower fixed doses for further group, it usually relies on the existence of mortality during the test. As described in guideline 420, application of the highest fixed dose, if only all above information for this classification of the test substance based on the identification of the dose which causing no mortality (OECD, 2001).

Regarding to Guideline 425, the difference is this guideline procedure uses single animals, with just beneath of the LD_{50} estimation to the first animal receiving a dose. The dose for the next whether increased or decreased is relying on the result of the previous animal and this order remains unchanged until there is difficulty of the initial outcome. A quantified number of animals are no longer dosed at the limit dose, there is no reversal before getting the selected higher (2000 or 5000 mg/kg) limit dose. Specific regulatory need only justifies the limit dose of 5000 mg/kg.

In short, Guideline 420 typically usually 1 animal would be predicted to die during the test, regards on Guideline 423 generally 2 to 3 animals per experiment may possibly be expected mortal in a complete experiment and last but not least Guideline 425 between 2 to 3 indicate the expected number of deaths.

2.2.2 Acute Oral Toxicity

When estimating the toxicity of mixtures and for risk assessment of environment and human health, acute oral toxicity data are used to satisfy hazard labelling and classification requirement. Classification for all regulatory authorities on establishment of either a point estimate of the range estimation or LD_{50} value generally complies with the acute oral toxicity data requirements (OECD 1999). A limit dose of 2000 mg/kg of the data is essential for the member countries for imposition. However, there are ranges of 2000 to 5000 mg/kg for substance with LD_{50} values in excess of 2000 mg/kg in few countries which have a requirement for information on toxicity at dose levels. Even though at doses of 2000 mg/kg or below, many establishments find it tolerable to use data from observations made, as defined in the GHS classification criteria, it is necessary for few regulatory authorities need the testing in this range. In addition, testing of animals in GHS category 5 ranges (2000-5000 mg/kg) is admonished for a reason of animal welfare concern, and should only be considered when the test has some reason for protecting human, animal health or the environment.

Acute toxicity testing can be defined as the adverse effect produces exposure in a short time to substance either by single or multiple exposures. Usually these adverse effects occur within 14 days of administration of any dangerous substances (UN, 2009). Practically, acute toxicity testing is different from chronic toxicity testing regarding about the time of exposure. An animal model is the best choice to do the acute toxicity study (UN, 2009). This is because it is unethical to use human as a test subject for acute toxicity study. In vitro method also has been more popular nowadays in order to reduce the amount of animal use in animal study.

Classification of five classes of acute toxicity hazard is established from three routes of acute toxicity such as oral, dermal or inhalation exposure route. The hazard categories of acute toxicity study have been simplified in Table 2.1 (UN, 2009). There is no evidence of this plant when perform experiments on its toxicity in water extract even though *P. minima* plant has been carried out in previous study on pharmacology. This method has been simplified by the OECD, (2000) and thus, in order to classify the hazard of the *P. minima*, oral acute toxicity study is the best choice.

Besides that, it is essential to know the time course for the development of toxicity signs testing. This is because when first-pass metabolism in animal is circulated via administration of chemical, the toxicity level can reduce due to biotransformation process (Wolansky, 2008). Rodents have become one of the most important animal models in the toxicology study. In rodents, an internal dose accumulation in nervous system tissue correlates with the onset of toxicity signs. According to Kelly *et al*. (2000), social behavior in rats has been shown to follow similar principles as in humans and also function of genetics, teratogenic influences, social learning and early mother infant interaction.

Table 2.1: Acute toxicity hazard categories based on LD_{50} range

Route of exposure	**Class 1**	**Class 2**	**Class 3**	**Class 4**	**Class 5**
Oral (mg/kg)	5	50	300	2000	5000
Dermal (mg/kg)	50	200	1000	2000	
Inhalation:					
Gases (ppm)	100	500	2500	5000	
Vapour (mg/l)	0.5	2.0	10.0	20	
Dust and mist (mg/l)	0.05	0.5	1.0	5	

UN, 2009

2.2.3 Rat's Typical Behaviour

The rat has been used a long time ago as an animal model for studies involving the nervous system either in toxicology or pharmacology. Rat strains are generally not easily genetically modified. Sprague Dawley rat is a type of rodent that is a multipurpose breed of white rat known with its calmness and ease of handling. Female rat is normally used in many toxicity studies rather than male rat (OECD, 2000). This is because the female generally slightly more sensitive. However, according to OECD (2001), when male shows more likely to be more sensitive based on information or knowledge of toxicology and toxicokinetics properties of related chemical, then this sex will be used.

Typical rat can perform grooming, socialized among groups, well intake of food and water and normal sleep pattern. They have performed good situation of locomotor activity includes walking, climbing, jumping, or crawling. The normal behaviour of rats once after walking is, first it will open eyes, rises and stretches. Then it will perform grooming by licking their back fur, lick hind toes, flanks and abdomen, scratches with them, and continuing repeating licks and scratching. Generally for the rat's behaviour, they will lick their tail, and genital, while hind toes scratches flanks and belly from front to back. Lastly, they will bite the fur (Brain, 1976). If one of this behaviour altered or changes, this might indicate that there is something wrong with the rat.

Besides, animal will show abnormal behaviour when there are in pain or stress condition. According to OECD (2000), pain can be defined as physical suffering or discomfort sensory and emotional torture that may cause potential tissue damage. There are three types of pain which are acute nociceptive pain, persistent inflammatory pain

and neuropathic pain. A sign that show by an animal when they are in pain can include evidence of infection, vocalization, avoidance of stimuli, or self-mutilation. While reduced in food intake might signal for chronic discomfort. Distress is suffering state result from inability or impact level of functioning to adapt to stress. Commonly, it is related with an alteration in physical functioning or motility and contributes to stereotype manners. Sniffing categorized as a component of exploratory behaviour. Through sniffing, the rats sample their surroundings by smell and whisker-touch. There are three clusters of movements, breathes in and out rapidly, secondly the rat probes its sound all over the place and along exteriors place, or thirdly the whiskers and the nose are in curt motion (Welker, 1964).

2.2.4 Clinical Sign of Acute Toxicity

In a document of OECD (2000), the behavioral sign of acute toxicity has been explained in detail and comprehensive. A variety of chemicals and drugs can alter behaviour via a multitude of mechanism of action (Weiner *et al*., 2009). However, it is too difficult to interpret, it as a primary index of neurotoxicity. Extemporaneous motor activity is defined as the willingness of the animal to move during the experiment because of the activities measured in the absence of any involvement like pharmacological or social. Motor activity refers to the foundation of actions, development and learning in response to any part of the body in either vertical or horizontal direction regardless of the distance travelled. There are two types of motor activity which are locomotors and non-locomotors movements. Locomotors movement refers to ambulation activity by alteration of the subject position within the experimental area either by walking, running, encircling, or rearing. While non-

locomotors activity related to behaviour such as grooming, burrowing, sniffing, pawing, body or head shakes and scratching. Changes in the pattern of motor activity result from any test substance administration, which efficiently amends all the interactions during dosing phase. Generally at sub-lethal doses, ample recovery of motor activity will occur within 8 to 10 hours in most cases.

2.2.5 Classification of Chemical Substances Based on Acute Toxicity

The classification of chemical substances based on acute toxicity has been established for the needs of existing systems. Generally, rats are preferred as test species to assess the acute toxicity by the oral and inhalation routes, while for acute dermal toxicity, rats or rabbits are preferred. Scientific judgement should be used to select the value of LD_{50} from valid, well performed test when the data of acute toxicity are available in several species. The acute toxicity hazard classification is shown in Table 2.1.

The highest toxicity category (Category 1) mainly used by the transport sector for classification for packing groups. Category 5 can be classified as relatively have low toxicity, but may cause a hazard to certain and susceptible populations. Criteria of chemical substances to fall into category 5 (oral or dermal toxicity test) are anticipated to possess LD_{50} value in the range of 2000-5000 mg/kg body weight and equivalent doses for inhalation toxicity test. Categories 5 possess higher doses, thus it is discouraged to perform on animals unless the test result has direct relevance to protect human health. It is essential to obtain information that allows the criteria to be applied for the classification determination. The classification of acute toxicity depends on the

amount of information available regarding the test item and its ingredients and the approach to the classification is tiered (UN, 2011).

2.2.6 Hazard Communication

One of the objectives of GHS is to implement harmonized hazard communication such as safety data sheets, labelling and symbols that are easily to be recognized and understand by consumers based on the classification criteria developed for the GHS. Hazard communication includes proper labelling tools to provide information regarding the categories and classes of hazards in GHS. The symbol of hazards, signal words and hazard statements for labelling of test items have been assigned and standardized based on the hazard categories. Hazard statement and communication is essential to distinguish the hazards according to the route of exposure (UN, 2011). All relevant classification and hazard communication should be implemented on the safety data sheet if the chemical substances or mixture (test item) is classified more than one route of exposure. It can also be distinguished based on the route of exposure if unknown acute (oral/dermal/inhalation) is communicated (UN, 2011). Specific label elements for test item are classified into acute toxicity Category 1 to 5 based on the criteria was shown in Table 2.2.

Table 2.2: Label elements for acute toxicity

	Category 1	Category 2	Category 3	Category 4	Category 5
Symbols	Skulls and crossbones 	Skulls and crossbones 	Skulls and crossbones 	Exclamation mark 	No symbol
Signal word	Danger	Danger	Danger	Warning	Warning
Hazard statement: --Oral	Fatal if swallowed	Fatal if swallowed	Toxic if swallowed	Harmful if swallowed	May be harmful if swallowed
--Dermal	Fatal in contact with skin	Fatal in contact with skin	Toxic in contact with skin	Harmful in contact with skin	May be harmful in contact with skin
--Inhalation	Fatal if inhaled	Fatal if inhaled	Toxic if inhaled	Harmful if inhaled	May be harmful if inhaled

2.2.7 Decision Logic

The GHS Acute Toxicity scheme has implemented five GHS categories based on the proper elements relevant to worker, consumer, transport and environment. Test item can be classified in GHS categories based on the LD_{50} (oral and dermal) and LC_{50} (inhalation). LC_{50} value is tested based on the observation of 4-hour test on animals (UN, 2011). The summarization of the five categories is shown in Table 2.3.

The most severe category which falls under Category 1, is mainly used by the transport sector for classification if packing groups. Some Competent Authorities may consider combining Acute Categories 1 and 2. The chemical substance that is relatively has low acute toxicity falls under Category 5 with certain conditions that is the test item might pose a hazard to vulnerable populations (UN, 2011). The identification of the substances in Category 5 can be determined based on the criteria mentioned in Table 2.3 besides LD_{50}/LC_{50}.

Table 2.3: Classification of acute toxicity substances based on GHS.

Acute toxicity	Category 1	Category 2	Category 3	Category 4	Category 5
Oral (mg/kg)	≤ 5	> 5 ≤ 50	> 50 ≤ 300	> 300 ≤ 2000	Criteria:- ● Anticipated oral LD_{50} between 2000 and 5000 mg/kg;
Dermal (mg/kg)	≤ 50	> 50 ≤ 200	> 200 ≤ 1000	> 1000 ≤ 2000	
Gases (ppm)	≤ 100	> 100 ≤ 500	> 500 ≤ 2500	> 2500 ≤ 5000	● Indication of significant effect in human; *
Vapors (mg/l)	≤ 0.5	0.5 ≤ 2.0	> 2.0 ≤ 10	> 10 ≤ 20	● Any mortality at class 4; *
Dust & mists (mg/l)	≤ 0.05	> 0.05 ≤ 0.5	> 0.5 ≤ 1.0	> 1.0 ≤ 5	● Significant clinical signs of class 4; * ● Indications from other studies. * * If assigned to a more hazardous class is not warranted

Decision logic provides additional guidance to classify the acute toxicity of test items. The decision logic of acute toxicity classification of substances is shown in Figure 2.1 and Figure 2.2 respectively. The classification of substances or mixtures in the class of acute toxicity and further allocation to a division is a very complex, five step procedure based on five GHS categories. The first step is to ascertain whether there is information regarding the evaluation of acute toxicity of the substance or mixture. The second step is the acceptance procedure and the third step is the assignment to the classification of acute toxicity of substances. The substance can be categorized accordingly based on the criteria of dosing amount with different routes of exposure (UN, 2011).

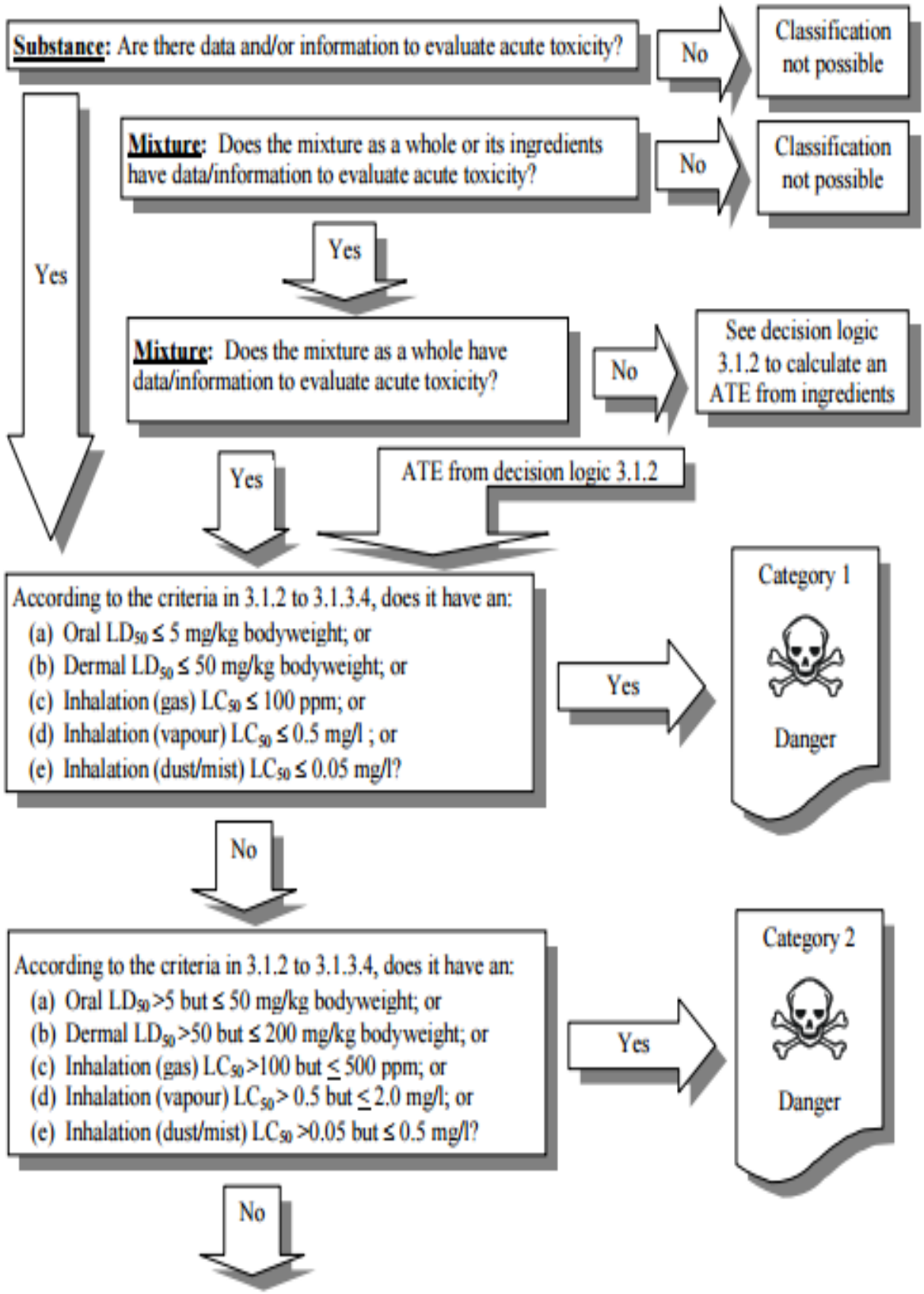

Figure 2.1: Decision logic of acute toxicity

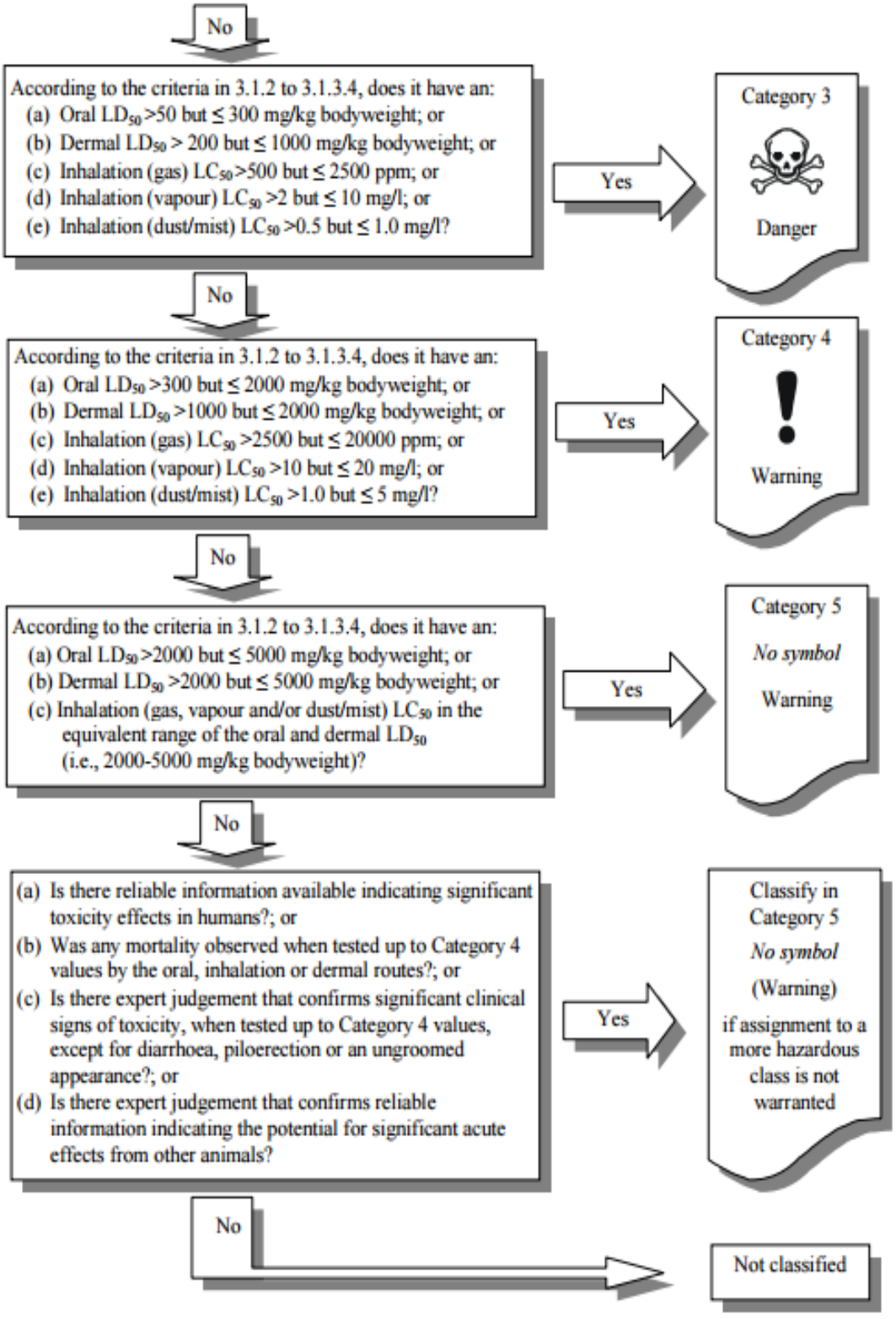

Figure 2.2: Continuity decision logic of acute toxicity.

2.3 Rat's Behaviours Assessment

Responses exhibit by animal to chemical material results from the communication of the substance with its cells, tissue and organs which may produce adverse effects that is expressed in behaviour. The behavioural changes caused by the toxicity of the chemical substance are recognized as the behavioural clinical signs. The OECD (2000) has listed types of interactive clinical signs that indicate the test animal is experiencing distress, severe pain or any abnormal locomotor function due to the toxicity of chemical substances. The animal should be humanely killed with the judgement of several signs that they exhibit, according to sufficient signals such as impending death, distress or severe pain. The following lists are the depiction of the clinical signs as well-defined by the OECD (2000), which are of interest in the study:

i. Abnormal discharge

Typically abnormal discharge might come from any external orifice, however rat normally will keep themselves hygienic.

ii. Ataxia/in coordination/staggering/unstable

This condition is due to weakness, post seizure recovery period, neuromuscular coordination and continuing observing the body weight.

iii. Blood surrounding the eyes and nose

Rodents could be experience blood surrounding to those organs, which is compulsory to distinguish between porphyrin secretion and blood. Commonly, secretion of porphyrin often related to a condition which the rodents experience in stresses and usually during

grooming it not secreted. However, if it is a blood, the end result presence may be a physical injury in nostril.

iv. Loss of bodyweight

Body weight loss or scrawniness is mostly when the rat's body weight declined within 20% to 25% compared with control animals over a period of 7 days or more. Usually it is accompanied by minimizing or absenteeism of food consumption.

v. Breathing difficulties

Hyperventilation, panting, abdominal-thoracic breathing and groaning with each breath is signs which can be presented by breathing difficulties (dyspnea).

vi. Body temperature

A lowered activity associated with any alteration in body temperature. Impending death commonly associated with where the body temperatures lower than 10% from normal temperature.

vii. Chromodachryorrhea

This behaviour may be accompanied by body weight loss and characterized by an animal going frequently making tracks in the cage. It indicates impaired of the inner ear or brain.

viii. Comatose

The animal may be in a state of deep unconsciousness for prolonged periods because too much lethargy, drowsiness due to adverse effects of the test material.