UNIVERSITI PUTRA MALAYSIA

TOXICOLOGY EVALUATIONS OF Rhaphidophora decursiva (Roxb.) Schott EXTRACT IN SPRAGUE DAWLEY

SITI SURIANI BINTI ARSAD

FPSK(M) 2013 45

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By

SITI SURIANI BINTI ARSAD

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Master of Science

October 2013

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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SITI SURIANI BINTI ARSAD

October 2013

Chairman : Norhaizan Mohd Esa, PhD

Faculty : Medicine and Health Sciences

Rhaphidophora decursiva (Roxb.) Schott has been widely used among Chinese community in Malaysia for treating colon cancer. The study aims to evaluate the toxic effects of the methanol extract of *R. decursiva* after single dose toxicity study (14-day acute toxicity study), repeated 28-day subacute toxicity and 90-day subchronic toxicity study in male Sprague Dawley rats, and also to determine the anti-proliferative activity of *R. decursiva* extract on colon cancer cell lines (HT-29) and normal cell lines (3T3). For toxicity study, rats were divided into 4 groups consisting of 6 rats per group for each acute, subacute and subchronic toxicity evaluations, with a total number of 72 rats. All control groups received distilled water (vehicle). For the acute toxicity, the 3 treatment groups received oral single dose of the plant extract at 700 mg/kg, 2800 mg/kg and 3500 mg/kg, respectively, and sacrificed at day 14 post administration of the plant extract. For subacute toxicity, the 3 treatment groups received daily oral dose of the plant extract at 70 mg/kg, 140 mg/kg and 210 mg/kg for 28 days. As no lethality was observed in subacute toxicity, similar doses were used for the 3 treatment groups in 90-day subchronic toxicity. The toxicity of *R. decursiva* extract was evaluated by the incident of lethality, cage side observations, body weight measurement, absolute and relative weight for kidneys and liver, hematological parameters such as erythrocyte count (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leucocyte count (WBC) and platelet count (Plt), then serum biochemistry parameters such as urea, creatinine (Crea), albumin (Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) and histopathological analysis of kidneys and liver. No lethality or adverse toxic signs were seen during the experimental periods in all toxicity studies. Behavior, body weight, absolute and relative organ weight also showed no significant changes in all three

toxicity studies. Hematological analyses revealed no statistically significant differences between groups in the studies. Serum biochemistry analyses showed no significant changes in acute and subchronic toxicity studies. Similar results were observed in subacute toxicity study. For histopathological findings, no significant changes ($p>0.05$) showed in liver tissue as well as kidneys tissues for all toxicity studies. Anti-proliferative activity of methanol extract of *R. decursiva* (Roxb.) Schott on HT-29 showed the highest sensitivity at 72 hours incubation period with IC_{50} value of 40 µg/mL, followed by 80 µg/mL and 260 µg/mL, respectively for 48 hours and 24 hours incubation period. Besides, the extract was found not to be cytotoxic on 3T3 (IC_{50} cannot be determined). In conclusion, based on clinical appearance, clinical pathological, organ pathology and histopathological results, the methanol extract of *R. decursiva* did not cause any toxic effects to male Sprague Dawley rats, and the oral lethal dose (LD_{50}) of the extract is more than 3500 mg/kg, while the no-observed-adverse-effect level (NOAEL) for the extract is 210 mg/kg per day for 90 days. Besides, according to the ability of *R. decursiva* extracts to achieve 50% inhibition of HT-29 cell lines, the findings from this study suggest that *R. decursiva* extract is safe to be consumed as no toxic effect was found when tested both in *in vivo* and *in vitro* studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Puta Malaysia sebagai memenuhi keperluan Ijazah Master Sains

PENILAIAN TOKSIKOLOGI EKSTRAK *Rhaphidophora decursiva* (Roxb.) Schott KE ATAS SPRAGUE DAWLEY

Oleh

SITI SURIANI BINTI ARSAD

Oktober 2013

Pengerusi : Norhaizan Mohd Esa, PhD

Fakulti : Perubatan dan Sains Kesihatan

Rhaphidophora decursiva (Roxb.) Schott telah digunakan secara meluas di kalangan masyarakat Cina di Malaysia untuk merawat kanser usus. Kajian ini dijalankan bertujuan untuk menilai kesan ketoksikan oleh ekstrak metanol tumbuhan selepas kajian toksik dos tunggal (kajian ketoksikan akut 14 hari), seterusnya berulang selama 28 hari (ketoksikan subakut) dan kajian ketoksikan subkronik selama 90 hari ke atas tikus jantan Sprague Dawley, serta menentukan anti-proliferatif aktiviti ke atas sel kanser usus (HT-29) dan normal sel (3T3). Untuk kajian tahap ketoksikan, tikus telah dibahagikan kepada 4 kumpulan yang terdiri daripada 6 ekor tikus untuk setiap kumpulan bagi setiap ujikaji ketoksikan akut, subakut dan subkronik, dengan jumlah bilangan 72 ekor tikus. Semua kumpulan kawalan menerima air suling (vehicle). Untuk ketoksikan akut, 3 kumpulan menerima dos tunggal dengan kepekatan ekstrak tumbuhan 700 mg/kg, 2800 mg/kg dan 3500 mg/kg, masing-masing, dan semua tikus tersebut dimatikan pada 14 hari selepas diberikan ekstrak tumbuhan secara oral (suapan). Bagi kajian ketoksikan subakut, 3 kumpulan yang telah diuji dengan menggunakan dos ekstrak tumbuhan pada kepekatan 70 mg/kg, 40 mg/kg dan 210 mg/kg selama 28 hari. Oleh kerana tiada kematian tikus semasa kajian ketoksikan subakut, maka dos yang sama telah digunakan untuk 3 kumpulan dalam ketoksikan subkronik (90 hari). Ketoksikan ekstrak *R. decursiva* telah dikaji melalui bilangan kematian tikus, pemerhatian bagi kelakuan tikus, pengukuran berat badan dan berat organ yang diambil seperti buah pinggang dan hati, keputusan hematologi seperti kiraan eritrosit (RBC), hemoglobin (Hb), hematokrit (Hct), isipadu kopuskular purata (MCV), hemoglobin kopuskular purata (MCH), konsentrasi hemoglobin kopuskular purata (MCHC), kiraan leukosit (WBC) dan kiraan platelet (Plt), seterusnya keputusan serum biokimia seperti urea, kreatinin (Cre), albumin (Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), dan alkaline phosphatase (ALP), dan analisis histopatologi ke atas tisu buah pinggang dan hati juga

dilakukan. Keputusan menunjukkan tiada kematian atau tanda-tanda toksik dilihat semasa tempoh eksperimen dalam semua kajian ketoksikan. Pemerhatian dari segi kelakuan, perubahan berat badan dan organ juga menunjukkan tiada sebarang perubahan ketara dalam tiga kajian ketoksikan ini. Hematologi analisis menunjukkan tiada perbezaan yang signifikan di antara kumpulan yang diuji dengan ekstrak *R. decursiva*. Serum analisis biokimia juga tidak menunjukkan sebarang perubahan yang signifikan dalam kajian ketoksikan akut dan subkronik. Keputusan yang sama telah diperhatikan dalam kajian ketoksikan subakut. Untuk keputusan histopatologi, tiada perubahan signifikan ($p>0.05$) diperhatikan dalam tisu buah pinggang serta tisu hati bagi semua kajian ketoksikan. Aktiviti anti-proliferatif *R. decursiva* (Roxb.) Schott ekstrak pada HT-29 menunjukkan kesan paling sensitif dengan nilai IC_{50} sebanyak 40 µg/mL selepas 72 jam tempoh pendedahan terhadap ekstrak. Manakala tempoh pendedahn ekstrak selama 48 jam menunjukkan kesan kurang sensitif ke atas sel HT-29 dengan nilai IC_{50} sebanyak 80 µg/mL diikuti dengan tempoh pendedahan ekstrak selama 24 jam menunjukkan kesan paling kurang sensitif dengan nilai IC_{50} yang tinggi iaitu 260 µg/mL. Sebaliknya, kajian yang dijalankan ke atas sel 3T3 menunjukkan ekstrak *R. decursiva* tidak memberi kesan toksik kerana tiada nilai IC_{50}. Kesimpulannya, berdasarkan keputusan klinikal darah, patologi organ dan histopatologi, ekstrak methanol *R. decursiva* tidak menyebabkan sebarang kesan toksik kepada tikus jantan Sprague Dawley, dan nilai 'lethal dose' (LD_{50}) ekstrak *R. decursiva* adalah lebih daripada 3500 mg/kg, manakala tahap 'no-observed-adverse-effect level' (NOAEL) bagi ekstrak *R. decursiva* ialah 210 mg/kg sehari selama 90 hari. Selain itu, mengikut keupayaan ekstrak *R. decursiva* untuk mencapai 50% perencatan terhadap sel HT-29, penemuan daripada kajian ini mencadangkan bahawa ekstrak *R. decursiva* adalah selamat untuk dimakan kerana tiada kesan toksik ditemui apabila diuji secara *in vivo* dan *in vitro*.

ACKNOWLEDGEMENTS

In the name of Allah, the most benevolent and most merciful. I would like to take this opportunity to thank all those who gave great support to me while doing this thesis. First and foremost, I would like to express my deepest gratitude to my main supervisor, Assoc. Prof. Dr. Norhaizan Mohd Esa for her generous guidance, patience, advice and endless support that contributed significantly towards the completion of this project. Her careful reviews and constructive criticism have been crucially important for this thesis.

My sincere gratitude is also accorded to my co-supervisors, Dr. Hazilawati Hamzah and Prof. Fauziah Othman for their constructive advice, priceless comment and invaluable advice throughout the entire course of this research.

Special thanks are expressed to the laboratoty staff of Department of Nutrition and Dietetics of Faculty of Medicine and Health Sciences, UPM especially, Mr. Syed Hasbullah Bin Syed Kamaruddin and Mr. Eddy Ghadaffie Bin Jamiauddin for their help, patience and guidance throughout my research study.

Finally, yet importantly, I would like to express my heartiest appreciation to all my family members for their understanding and support throughout my studies. Also, thank you to all my friends and laboratory mates for support, help and guidance.

Above all, I thank God for His mercy and blessing on me.

I certify that a Thesis Examination Committee has met on 29 October 2013 to conduct the final examination of Siti Suriani binti Arsad on her thesis entitled "Toxicology Evaluations of *Rhaphidophora decursiva* (Roxb.) Schott Extract in Sprague Dawley" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Asmah binti Rahmat, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Sharida binti Fakurazi, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Loh Su Peng, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Nor Fadilah Rajab, PhD
Associate Professor
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)

NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 January 2013

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree. The members of the Supervisor Committee are as follows:

Norhaizan Mohd Esa, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Fauziah Othman, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Hazilawati Hamzah, PhD
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
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Signature:	_____	Signature:	_____
Name of Chairman of Supervisory Committee:	Associate Professor Dr. Norhaizan Mohd Esa	Name of Member of Supervisory Committee:	Professor Dr. Fauziah Othman
Signature:	_____		
Name of Member of Supervisory Committee:	Dr. Hazilawati Hamzah		

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µL	microlitre
µmol/L	micromole per litre
ACF	aberrant crypt foci
ACS	American Cancer Society
ACUC	Animal Care Use Committee
AIDS	Acquired immunodeficiency syndrome
Alb	albumin
ALP	alkaline phosphate
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CC	cellular cast
CM	conservative medicine
CO_2	carbon dioxide
Cre	creatinine
DHI	5,6- dihydroxyindole
dL	decilitre
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic Acid
EEC	European Economic Community
FBS	fetal bovine serum
fL	femtoliters
FRIM	Forest Research Institute of Malaysia
GHS	Globally Harmonized System
g	gram
g/L	gram per litre
GC	granular cast
Hb	hemoglobin
Hct	hematocrit
CPV	cytoplasmic vacuolation
IARC	International Agency for Research on Cancer
L	litre
LD_{50}	50% lethal dose
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
mg/kg	milligram/kilogram
mL	mililitre
mmol/L	milimole per litre

MTT	(3-[4,5-dimethylthiazol-2-yl]-2-5-diphenytetrazoliumbrimide)
NCR	National Cancer Registry
NCSM	National Cancer Society of Malaysia
NCCAM	National Center for Complementary and Alternative Medicine
NOAEL	no-observable adverse effect levels
NOEL	no-observable effect
OECD	Organization for Economic Cooperation and Development
PBS	phosphate buffer saline
P	protein cast
Pg	picogram
Plt	platelet count
RBC	red blood cell
RDW	red blood cell distribution width
Rpm	revolutions per minute
RPMI	Rosswell Park Memorial Institute
S.E.M	standard error of mean
SARS	Severe Acute Respiratory Syndrome
SPSS	Statistical Package of Social Science
SW	sinusodial widening
U/L	units/litre
UK	United Kingdom
UKM	Universiti Kebangsaan Malaysia
UMS	Universiti Malaysia Sarawak
UNESCO	United Nations Educational, Scientific and Cultural Organization
UPM	Universiti Putra Malaysia
UTM	Universiti Teknologi Malaysia
USA	United State of America
USM	Universiti Sains Malaysia
WBC	white blood cell
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 **Background**

Plants and herbs have been used since the earliest of times to cure various ailments. By the middle of the nineteenth century, about 80% of all medicines were derived from herbs. Generally, herbs refer to yearly, seasonal or biennial seed-producing, non-woody plants that die at the end of each harvest season. The National Center for Complementary and Alternative Medicine (NCCAM) (2011) defined herb as a plant or part of a plant that is used for its smell, taste or healing characteristics. The flowers, stems, seeds or roots of a plant can bring benefits in medical or aromatherapy attributes. McCann (2004) stated that generally, health care experts acknowledge herbs as a raw drug that can be used to treat infections, avoid illnesses or sustain good health. Nonetheless, herbs can be made into extracts, drinks, teas, pills, oils and shampoos.

McCann (2003) also reported that herbs have significance in current day biomedical antidotes. In a quarter of biomedical medications regularly prescribed nowadays, at least one active ingredient comes from plants and the rest of the substances are chemically produced in laboratories. Many medical herbs have long been used as anticancer agents (Tiwari *et al*. 2000; Duong Van Huyen *et al*. 2003). Recent evidence from studies by Prozeski *et al*., (2001), Kawada *et al*. (2001) and Moalic *et al*. (2001) reported that a number of natural products originated from plants have antitumor properties. Besides this, a study by Wojdylo *et al*. (2007) has also shown that herbs promote antioxidant activity, digestion, anti-inflammatory, anti-microbial, prevent genetic mutation effects and have anti-cancer agents.

Herbal medicine are substance that come from one or more plants and brings healing qualities or health benefits to human in forms of raw or processed ingredients. Herbal medicine as the major remedy in traditional medical systems, have been used in medical practice for thousands of years and have made a great contribution to maintain human health. World Health Organisation (WHO) (1993) informed that a majority of the world's population in developing nations still depend on herbal medicines to fulfill its wellbeing needs. A study by MacManus (2008) has shown that many herbal medicines are effective since they contain pharmacologically active substance derived from plants. Li *et al.* (2009) explained that extracts from the herbs are often used in line with long-established cancer treatments to improve survival rate and quality of life because it is found to be cheaper than conventional treatments. Ali *et al.* (2005) disclosed that in Malaysia and Indonesia, factory-made traditional herbal antidotes such as the popular 'jamu' and 'makjun' are easily available and taken in frequently, up to 3 times daily to promote good health. Furthermore, the Indonesian 'jamu' is also available in Singapore, Australia and Holland where there is a large community of Indonesians (Ali *et al.* 2005).

Joshi & Kaul (2001) further added that traditional herbal medicines are gaining popularity globally and these products purchases have raised significantly. This is due to the fact that research has reported that essences from several herbal medicines or blend have an anticancer potential *in vitro* or *in vivo*. Various extracts either water extract or alcohol extract of herbs that are used by researchers have shown that there are some anti-proliferative effect on cancer cell lines. Instead, earlier studies also have shown that there are some antioxidant mechanisms in herbs that act as an anticancer agent (Prasad *et al.* 1999). However currently, poison related to herbal drugs is becoming more of a concern in terms of their increasing intake in developed nations. WHO (2000) and International Agency for Research on Cancer (IARC) (2002) pointed out that appropriate instructions, guidelines and assessment have been published to maintain the safe practice of these products. Saad *et al.* (2006) revealed that the usage of a number of herbal and herbal derived goods could result in a hepatic toxicity. Thus, herbal scientists or manufacturers must understand the association between pharmacological reactions and the chances of the herbs dynamic compounds interacting with other drugs when both are taken together.

Timbrell (2000) put forward that researches in toxicology play a significant role in pharmaceutical sciences in which it uncovers the needed comprehension on how the human body works and drugs interactions within it. Salsburg (1986) defined the LD_{50} value as the approximation of the potency of toxicant or poisons to subjects which results in fifty percent of lethality and generally stated in mg/kg body weight. However, other conditions such as growth of cancer and organ damage resulted from substances are not measured. This is because there is doubt in the forecast of other outcomes such as change of enzyme activity and organ impairment based on the LD_{50} value. A comprehensive evaluation of blood serum tests such as liver and kidney function test, hematology and histopathology, food and water intake need to be performed on subjects to investigate the occurrence of secondary stage of toxicity resulted by chemicals. Information from a toxicity study will be used as reference for the selection of doses for repeated-dose study, presenting initial detection of target organ toxicity or any contrary effects after consumption.

Rhaphidophora decursiva (Roxb.) Schott is used widely in the herbal and traditional medicinal preparations especially among the Chinese community. They believe that this plant can be used to treat colon cancer and they commonly consumed it in the form of herbal tea. However, there are still no scientific studies that have been done to prove the effectiveness of this plant in treating colon cancer. Therefore, many studies need to be done especially in terms of toxicity studies before it can be promoted for utilization or be commercialized. Information from a toxicity study will be a useful tool for the indication of doses for repeated-dose study, offering initial detection of target organ toxicity or any harmful effects after use.

1.2 Problem statements

Herbal medicines have been widely utilized as effective remedies for the prevention and treatment of multiple health conditions for centuries by almost every known culture. The first documented records of herbal medicine use date back 5,000 years (NCCAM, 2005) in China. Similarly, India's Ayurvedic medicine tradition is thought to be more than 5,000 years old and herbal medicine remain an essential component of its practice (Astin *et al.* 2000). Today, the populations of certain countries still depend on herbal medicines to address their healthcare needs.

Worldwide it is estimated that 80% of the population uses herbs; in the developing world rates could be as high as 95% (Nahin *et al.* 2007). WHO estimates that the global market is approximately US $83 billion annually (Nahin *et al.* 2007).The use of herbal medicines continues to expand globally, in parallel to an increasing acceptance of herbal remedies by consumers. Despite the fact that herbal remedies are not classified as drugs by the United States (US) Food and Drug Administration (FDA) Dietary Supplements Health and Education Act, 1994, the attitude of the general population toward herbal medicine is that this kind of therapy is natural and therefore safe (Zaffani *et al.* 2006). So despite the potential for harmful side effects (De Smet, 1995) and interactions with conventional drugs (Williamson, 2003; Chavez *et al.* 2006), natural products are often taken on a self-medication basis, without the advice of pharmacists or physicians (Eisenberg *et al.* 1998). The safety and efficacy of many herbal medicines used remain essentially unknown. There are limited clinical trials to determine efficacy and safety of traditional herbal medicines. This lack of research does not impede most from using them, given that these remedies are often grounded in long standing cultural traditions.

Similarly, Chinese community in Malaysia believes that *R. decursiva* is effective in curing colon cancer but there is also no scientific researches have been done before. In prior research, it was found that *R. decursiva* actually contains antimalarial compounds by performing antimalarial bioassay-directed fractionation, which has led to the isolation of 'decursivine', a new active indole alkaloid, from the leaves and stems of *R.decursiva* (Zhang *et al.* 2002). There are possibilities of toxicity effect present due to the long term use and unpredictable amounts of the substance that produces the therapeutic effect (Li *et al.* 2009). Saper *et al.* (2008) reported that 20% of Ayurvedic medicines purchased via the Internet contained detectable levels of lead, mercury, and arsenic. Herbs that have caused major adverse events include creosote bush (hepatotoxicity) (Slade & Keating, 2007) and kava (hepatotoxicity) (Kwon *et al.* 2006). Using the proper parts of the plant and the appropriate process for obtaining the ingredients could prevent toxicity, as seen in kava-induced toxicity (White *et al.* 2007).

1.3 Significant of the study

Natural products, especially plants, have been used for the treatment of various diseases for thousands of years (Shoeb, 2006). Although many research-related medicinal plants have been made over the past 30 years, a large number of plants still have not been studied (Kakuko *et al.* 2005). Malaysia have various kind of plants used for medicinal purposes. There are lots of herb plants surviving in the Malaysian rain forest. Some familiar and unfamiliar species allow the researchers to study more in terms of their beneficial aspect to human health.

The substance of the organic components as well as the effects such as anti-inflammatory and anticancer activities in plants may differ. Wu *et al.* (2002) stated that some studies revealed that substances in natural products bring positive outcomes in cancer treatment when weighed against with chemotherapy or existing hormonal therapies. Furthermore, Lee *et al.* (2004) added that specifically, oriental therapeutic plants are believed to be one of the main potential sources due to their variation in types and purposes.

Nowadays, most research has focused on certain plant species that have the potential benefits to the human body, and until now, many other species under the same family group also have not been undiscovered as to bringing beneficial effect on health. For instance, some studies have discovered the potential of Rhaphidophora species that belong to the Araecea family in the prevention and curative abilities for certain health problems. This species may include *Rhaphidophora korthalsii*, *Rhaphidophora glauca*, *Rhaphidophora elliptica*, *Rhaphidophora cryptantha*, *Rhaphidophora pertusa*, *Rhaphidophora decursiva*, *Rhaphidophora hookeri*, *Raphidophora hongkongensis*, and many more. Each of them has their own biological active components that play an important role as a curing agent for certain health problems. To date, only a few studies have focused on this species since they are rarely found and not yet commercialized including *R. decursiva* which is also known as Pa Shu Long or Shan Shu Long. This plant can be grouped as unfamiliar species that needs to be investigated for their bioactive compound.

In view of the complexity of herbal medicines and their inherent biological variations, it is necessary to evaluate their safety, efficacy, and quality (Castro *et al.* 2009). With the increasing usage of herbal therapies, significant concerns have been raised over the lack of quality control and scientific evidence of the efficacy and safety of these agents (Firenzuoli & Gori, 2007).In particular, safety of herbal prescriptions has become an increasingly important issue (Tang *et al.* 2008) and is generally evaluated via toxicological assessment of all medicinal plant materials. *R. decursiva* which is rarely known among people will increase in value due to the known safety dosage through this study and it can be used for further research especially anticancer study.

1.4 Objectives

1.4.1 General objective

To study the toxicology evaluations of *Rhaphidophora decursiva* (Roxb.) Schott extract on Sprague Dawley rats.

1.4.2 Specific objectives

1. To determine the effect of doses and time on physical haematological, biochemical and histopathological of liver and renal functions.

2. To evaluate the toxicological potential of the plant extract on liver and kidney.

3. To determine the IC_{50} value of *Rhaphidophora decursiva* (Roxb.) Schott extract on the proliferation of normal cell line (3T3) and colon cancer (HT-29) cell line.

Limitations of the study

In the light of the fact that this plant can be found in different regions of the country as well as in other countries, however the plant samples that were used in this study were only collected in one selected region in Malaysia which may not represent the same species of the plant found in other parts of the world and thus, cannot be generalized by other researchers. Besides this, the plant samples may not represent the whole part of the plant because the study was only carried out on the leaves part. Therefore, the assumptions for the effect of the whole plant cannot be obtained. In addition to this, there are several aspects that may affect the quality of the samples which may include the transportation, processing and storage that had caused the samples not to be really fresh.

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