IN VITRO CYTOTOXICITY ACTIVITY AND *IN VIVO* ORAL TOXICITY OF *Euphorbia hirta*

KWAN YUET PING

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by

KWAN YUET PING

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KWAN YUET PING

Institute for Research in Molecular Medicine

Universiti Sains Malaysia

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
DCFH-DA	2', 7'-Dichlorodihydrofluorescin diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
E. hirta	*Euphorbia hirta*
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FTIR	Fourier Transform Infrared
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory Concentration 50%
LC-MS	Liquid Chromatography-Mass Spectrometry
LD_{50}	Lethal Dose 50%
MPLC	Medium Pressure Liquid Chromatography
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
RPMI 1640	Rosswell Park Memorial Institute
RNAse	Ribonuclease
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SPSS	Statistical Package for the Social Sciences
WBC	White Blood Cell count
WHO	World Health Organisation

AKTIVITI SITOTOKSIKAN SECARA *IN VITRO* DAN UJIAN KETOKSIKAN ORAL SECARA *IN VIVO* DARIPADA *Euphorbia hirta*

ABSTRAK

Euphorbia hirta adalah sejenis tumbuhan yang digunakan dalam perubatan tradisional secara meluas untuk mengubati pelbagai jenis penyakit. Sitotoksik, genotoksik secara *in vitro* dan penilaian ketoksikan secara *in vivo* ekstrak metanol daripada *E. hirta* telah diselidik dalam kajian ini. Penilaian pelbagai parameter farmakognosi telah dijalankan mengikut garis panduan Pertubuhan Kesihatan Sedunia (WHO) untuk pempiawaian ekstrak *E. hirta*. Pempiawaian kandungan kimia melibatkan kuantifikasi sebatian kimia utama dalam ekstrak *E. hirta*. Kaedah GC-MS yang telah digunakan untuk kuantifikasi asid 1,3,4,5 tetrahidroksisikloheksanakarbosilik dalam ekstrak adalah cepat, tepat, dan linear. Enam puncak utama dalam lingkungan 600 - 1500 dan 2800 - 3400 cm^{-1} diperhatikan dalam spektrum FTIR. Kepekatan logam berat dalam ekstrak *E. hirta* adalah didapati berada di bawah had yang dibenarkan. Dalam asai antioksidan terhadap radikal bebas DPPH, ekstrak *E. hirta* menunjukkan aktiviti antioksidan 50% ke atas, tidak bergantung pada masa pengekstrakan. Kajian potensi ketoksikan ekstrak *E. hirta* telah dilakukan dengan menggunakan kaedah kematian anak udang dan ujian sitotoksisiti terhadap sel-sel kanser. Ekstrak *E. hirta* menunjukkan kesan toksik yang ketara terhadap anak udang dengan nilai (kepekatan maut) LC_{50} sebanyak 620.38 µg/mL (24 jam). Perbandingan keputusan ini dengan kesan toksik kawalan positif iaitu kalium dikromat didapati bahawa kesan aktiviti ketoksikan yang ditunjukkan oleh ekstrak metanol adalah sederhana. Ujian sitotoksisiti MTT

menunjukkan bahawa ekstrak *E. hirta* merencatkan pertumbuhan sel MCF-7 dengan nilai (kepekatan perencatan) IC_{50} sebanyak 25.26 µg/mL yang semakin meningkat seiring dengan peningkatan kepekatan ekstrak dan masa pengeraman. Kajian mikroskopik menunjukkan bahawa sel-sel yang dirawat dengan ekstrak *E. hirta* menandakan ciri-ciri morfologi apoptosis. Ekstrak *E. hirta* juga dikesan tidak mempunyai pengaruh terhadap kebocoran laktat dehidrogenase (LDH) dan penghasilan species oksigen reaktif (ROS) secara intrasel. Oleh itu, *E. hirta* mungkin merangsang kesan sitotoksisiti melalui mekanisma yang tidak bersandarkan kepada ROS terhadap sel-sel MCF-7. Analisis sitometri aliran dengan pewarnaan Annesin V/Propidium Iodida mengesahkan bahawa ekstrak *E. hirta* merangsang apoptosis dalam sel-sel MCF-7. Rawatan ekstrak *E. hirta* juga menyebabkan fragmentasi DNA dalam sel-sel MCF-7. Tambahan pula, rawatan *E. hirta* menyebabkan pengumpulan sel-sel di fasa S and G_2/M kitaran sel dengan menginduksi apoptosis. Kajian aktiviti kaspase mendedahkan bahawa ekstrak *E. hirta* mengaruhi apoptosis tanpa bersandarkan laluan kaspase-3 tetapi melalui pengaktifan kaspase-2, 6, 8 dan 9. Untuk mengenal pasti sebatian kimia yang bersifat sitotoksiti, penyisihan ekstrak *E. hirta* telah dilakukan berpandukan bioasai. Subfraksi heksan *E. hirta* iaitu EH Hex 4 menunjukkan aktiviti sitotoksiti yang signifikan di kalangan semua fraksi yang diuji. Analisis selanjutnya dengan menggunakan kaedah LC-MS terhadap fraksi EH Hex 4 membawa kepada identifikasi sebatian 3,3',4',5,7-Pentahydroxy-8-(5-oxo-2-pyrrolidinyl) flavone sebagai agen cenderung kepada sitotoksik di dalam ekstrak *E. hirta*. Data yang diperolehi daripada kajian ini menunjukkan bahawa *E. hirta* menyebabkan kematian sel secara apoptotik dan mencadangkan bahawa *E. hirta* boleh digunakan sebagai agen anti-kanser yang mengaruhi apoptosis untuk rawatan kanser payudara dengan kajian lanjutan yang terperinci. Kesan potensi genotoksik

E. hirta telah dikaji dengan menggunakan ujian *Allium cepa* and asai komet. Dalam ujian *A. cepa*, ekstrak *E. hirta* menunjukkan kesan genotoksik dan mitodepresif yang signifikan pada kepekatan sebanyak 1000 µg/mL.. Peningkatan dalam nilai aberasi kromosom yang bersandarkan kepada kepekatan ekstrak telah diperhatikan seperti kromosom kelekitan, kromosom c-mitosis, kromosom jambatan dan kromosom vagrant. Sel mikronukleus juga telah diperhatikan pada interfasa. Dalam ujian komet, rawatan dengan 25 µg/mL ekstrak *E. hirta* selama 72 jam pada sel-sel MCF-7 menyebabkan peningkatan kerosakan DNA sebanyak 48.16% berbanding dengan kawalan yang tidak dirawat. Data ini mengesahkan kesan genotoksik ekstrak *E. hirta*. Ujian ketoksikan oral akut dan sub-kronik ekstrak *E. hirta* telah dilakukan dengan menggunakan tikus Sprague Dawley. Ekstrak pada dos tunggal iaitu sebanyak 5000 mg/kg berat badan tidak menunjukkan kematian atau kesan toksik yang berkaitan dengan rawatan tersebut dalam tempoh pemerhatian selama 14 hari. Oleh itu, dos LD_{50} (dos membunuh) tumbuhan ini dianggarkan melebihi 5000 mg/kg. Dalam kajian ketoksikan sub-kronik, pemberian ekstrak *E. hirta* sebanyak 50 mg/kg, 250 mg/kg, 1000 mg/kg/hari berat badan menunjukkan tiada sebarang perbezaan yang signifikan dalam makanan dan penggunaan air, perubahan berat badan, parameter hematologi dan biokimia, berat relatif organ dan pemerhatian secara kasar berbanding dengan kumpulan kawalan. Pemeriksaan makropatologi dan histopatologi kesemua organ tidak menunjukkan sebarang perubahan morfologi. Penganalisaan keputusan ini bersama-sama dengan informasi mengenai tanda-tanda penyakit, tingkah laku dan pemantauan kesihatan boleh membawa kepada kesimpulan bahawa pengambilan jangka panjang ekstrak *E. hirta* selama 90 hari tidak menyebabkan ketoksikan sub-kronik. Kesimpulannya, keputusan ujian ketoksikan secara *in vitro* dan *in vivo* yang diperolehi dalam kajian ini membuktikan

tentang kepentingan kedua-dua kaedah tersebut untuk memperolehi data penilaian

toksikologi yang komprehensif untuk agen terapeutik semula jadi.

IN VITRO CYTOTOXICITY ACTIVITY AND *IN VIVO* ACUTE ORAL TOXICITY OF *Euphorbia hirta*

ABSTRACT

Euphorbia hirta is an annual plant that has been widely used in traditional medicine to treat various diseases. *In vitro* cytotoxicity, genotoxicity properties and *in vivo* toxicological evaluation of *E. hirta* methanol extract were investigated in this study. Various pharmacognostical parameters evaluation was carried out as per World Health Organization (WHO) guidelines procedure for the standardization of *E. hirta* extract. The chemical constituent aspect of standardization involves quantification of the main chemical components in *E. hirta*. The GC-MS analysis was used for quantification of 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid in the extract was rapid, accurate and linear. Six major peaks in the range of 600 - 1500 and 2800 - 3400 cm^{-1} were observed in the FTIR spectra. The concentrations of heavy metals determined in *E. hirta* extract were well below the permissible limit. In 2,2-Diphenyl-1-picylhydrazyl radical (DPPH) assay, the extract showed a general consistency on antioxidant activity above 50%, independent of the extraction time. The potential cytotoxicity effect of *E. hirta* was investigated using brine shrimp lethality assay and the cytotoxicity assay against cancer cells. The extract of *E. hirta* showed significant toxicity against brine shrimp with (Lethal Concentration) LC_{50} value of 620.38µg/mL (24 h). Comparison with positive control potassium dichromate signifies that cytotoxicity exhibited by the methanol extract have moderate activity. The 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay showed that *E. hirta* inhibited MCF-7 cell

viability with the (Inhibitory Concentration) IC_{50} values 25.26 µg/ml in a dose and time-dependent manner. Microscopic studies showed that *E. hirta* treated cells exhibited marked morphological features characteristic of apoptosis. *E. hirta* extract also had an ignorable influence on the lactate dehydrogenase (LDH) leakage and generating intracellular reactive oxygen species (ROS). Therefore, *E. hirta* might induce the cytotoxicity via a ROS-independent mechanism in MCF-7 cells. The Annexin V/Propidium Iodide flow cytometry study confirmed that *E. hirta* extract induced apoptosis in MCF-7 cells. *E. hirta* extract treatment also resulted in DNA fragmentation in MCF-7 cells. Moreover, *E. hirta* treatment resulted in the accumulation of cells at the S and G_2/M phases as well as apoptosis. The caspase activity study revealed that *E. hirta* extract induced apoptosis through the caspase-3 independent pathway by the activation of caspase-2, 6, 8 and 9. To identify the cytotoxic compound, *E. hirta* extract was subjected to bioassay-guided fractionation. *E. hirta* hexane subfraction, namely EH Hex 4 demonstrated highest activity among all the fractions tested. Further Liquid Chromatography–Mass Spectrometry (LC-MS) analysis of EH Hex 4 led to identification of 3,3',4',5,7-Pentahydroxy-8-(5-oxo-2-pyrrolidinyl) flavone as the likely cytotoxic agent in *E. hirta* extract. The data obtained from this study revealed that *E. hirta* induced apoptotic cell death and suggests that *E. hirta* could be used as an apoptosis-inducing anti-cancer agent for breast cancer treatment with further detailed studies. The potential genotoxic effect of *E. hirta* was investigated using *Allium cepa* and comet assay. In *A. cepa* assay, the result showed that the methanol extract of *E. hirta* exerted significant genotoxic and mitodepressive effects at 1000 µg/mL. A dose-dependent increase of chromosome aberrations was also observed such as stickiness, c-mitosis, bridges and vagrant chromosomes. Micronucleated cells were also observed at interphase. In the comet

assay, the treatment of 25 µg/mL of *E. hirta* extract for 72 h on MCF-7 cells caused an increase in DNA damage by approximately 48.16% compared to the unchallenged control which confirmed the genotoxic effect of *E. hirta* extract. The acute and sub-chronic oral toxicity of *E. hirta* was evaluated in Sprague Dawley rats. The extract at a single dose of 5000 mg/kg did not produce treatment related signs of toxicity or mortality in the animals tested during the 14-day observation period. Therefore, the (Lethal Dose) LD_{50} of this plant was estimated to be more than 5000 mg/kg. In the subchronic toxicity study, the administration of 50 mg/kg, 250 mg/kg, 1000 mg/kg/day of *E. hirta* extract per body weight revealed no significant difference in the body weight change, haematological and biochemical parameters, relative organ weights and gross findings compared to the control group. Macropathology and histopathology examination of all organs did not reveal morphological alteration. Analyses of these results with the information of signs, behaviour and health monitoring could lead to the conclusion that the long-term oral administration of *E. hirta* extract for 90 days does not cause toxicity. In conclusion, *in vitro* and *in vivo* toxicity results obtained in this study proved the importance of both methods to obtain comprehensive toxicological evaluation of natural product agents.

CHAPTER 1.0: INTRODUCTION

1.1 Overview and rationale of the study

Natural products, especially medicinal plants, have played a significant role in drug discovery and development of therapeutic agents. Plants contain many biologically active compound(s) which have potential for development as therapeutic agents. More than 35,000 plant species have been reported to be used in various human cultures around the world for medical purposes (Lewington, 1993; Faleyimu and Oso, 2012). There is a growing scientific consensus about the impending loss of tropical species as some of these species may not be available to future generations of natural product drug discovery scientists (Gurib-Fakim, 2006). Plants of the tropical rainforests especially in Malaysia serve as a potential source of pharmaceutical leads due to the high chemical diversity. Hence, toxicity studies play an important role in the identification and isolation of pharmaceutical leads from medicinal plants.

Although *Euphorbia hirta* (*E. hirta*) has been used for generations as a medicinal herb, however little is known of the toxicological potential of the herb. Based on their long-term use by humans, one might expect *E. hirta* used in traditional medicine to have low toxicity. However, recent research have revealed that many plants used as food or in traditional medicine showed toxic effects in *in vitro* assays (Cardoso *et al.,* 2006; Mohd-Fuat *et al.,* 2007). This raises concern about the potential toxic hazards resulting from the short-term and long-term use of *E. hirta*. Therefore, evaluating the toxicological effects of *E. hirta* extract intended to be used in humans is a crucial part of its assessment for potential hazards. Regarding drug discovery and development, there are different weights of interest of all

concerned groups, which need to be taken into consideration (Olejniczak *et al*., 2001). The general public, patients and consumers are mostly interested in the fast access to safe and efficient medicines, as well as in animal welfare. Health authorities are obliged to make sure that laws and regulations are followed, i.e., both regulatory laws on medicines and on animal protection.

Moreover, the pharmaceutical industry plans to produce safe and ethical medicine in the most economic way. The different interests of the public, the pharmaceutical industry and health authorities may lead to potential conflicts. Therefore, the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use), the common regulatory initiative of the EU, USA and Japan has addressed these topics in their mission and goals, which are: to provide and assure safe and ethical medicines; to maintain regulatory compliance; to achieve fast development and market authorization; to economize costs and to consider animal protection (ICH, 2007). In light of the given situation of therapeutic agents development and because of the above described interests, new approaches are progressing in different phases of drug development that allow for more efficient, yet safe procedures. One possible way to economize time and costs, as well as to address animal protection, is to introduce alternative methods into non-clinical safety testing. Currently, animal tests are mandatory for the evaluation of acute toxicity of chemicals and new drugs. The replacement of the *in vivo* tests by alternative *in vitro* assays would offer the opportunity to screen and assess numerous plants extract at the same time, to predict acute oral toxicity and thus accelerate drug development processes. Furthermore, the substitution of *in vivo* tests by *in vitro* methods shows a proactive pursuit of ethical

and animal welfare issues. There is little toxicological data available on *E. hirta* although the plant has been used as herbal medicine since ancient time. Hence, the current study was designed to compare the *in vivo* and *in vitro* toxicity of *E. hirta* extract besides evaluating its potential as anticancer agents and its mechanism of actions. The conflict between *in vitro* and *in vivo* studies persists as the results obtained from *in vitro* studies are often not directly interpretable in the context of potential *in vivo* exposures (Yoon *et al.,* 2012). *In vitro* data from cell-based assays should be viewed as indicators of potential toxicity, but not as absolute markers. On the other hand, *in vivo* data from animal studies are more indicative of toxicity and may be considered to be safety markers (WHO, 2000).

Cancer is a growing health problem around the world characterised by uncontrolled cell growth. Breast cancer is the one of the most common cancer in females. Global breast cancer case has increased from 1.38 million women in 2008 to more than 1.6 million in 2010 (Ferlay *et al*., 2012). There were 3242 female breast cancer cases diagnosed in 2007, accounted for 18.1% of all cancer cases reported and 32.1% of all female cases (Zainal and Nor Saleha, 2011). It is estimated over 60% of anticancer drugs available in the market are of natural origin and majority of these compounds are obtained from higher plants (Newmann *et al.*, 2003). The development of anticancer drugs, especially cytotoxic agents from medicinal plants, differs significantly from the drug development process for any other indication. Major research efforts are aimed at discovery of molecular targets that are specific for cancer cells, development of agents that are toxic to cancer cells, and at devising their synergistic combinations. Hence, the identification toxicity of medicinal plants may contributes for the identification of medicinal plants that are selectively induces

cytotoxicity in cancer cells but not in normal cells, which is an unusual property that is not shared by conventional chemotherapeutic agents (Blagosklonny and Pardee, 2001; Li *et al*., 2003). The development of anticancer drugs from natural sources requires toxicity information on the constituent of interest. It should be emphasized that toxic effects of the anticancer agent on the host cells must be considered, as a substance may exhibit an apparent anticancer activity by virtue of its toxic effect on the cells. Therefore, in this study besides various toxicity evaluation of *E. hirta* extract, the anticancer potential of this plant on cancer cells was also evaluated to develop therapeutic agents which selectively toxic (toxicity to cancer cells but not to normal cells) on cancer cells.

1.2 Research objectives

The current study was undertaken with the following objectives:

1. To standardize the methanolic extract of *E. hirta*

2. To determine the *in vitro* cytotoxicity of methanolic extract of *E. hirta* on several cell lines and *Artemia salina*

3. To determine the mode and mechanism of cell death in MCF-7 cells treated with methanolic extract of *E. hirta*

4. To determine the genotoxicity of methanolic extract of *E. hirta*

5. To evaluate *in vivo* acute and sub-chronic oral toxicity of *E. hirta* extract administration in albino rats

6. To identify cytotoxic fraction/compound (s) from *E. hirta*

CHAPTER 2.0: LITERATURE REVIEW

2.1 Pharmaceuticals from plants

2.1.1 Background of herbal medicine in Malaysia

From prehistoric times, plants have been a valuable source of natural products for treatment of various diseases. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's population relying mainly on traditional medicines for their primary modality of health care (Farnsworth *et al.*, 1985; Jyothi *et al*., 2011).

Malaysia is among the world's top 12 rich biodiversity countries where Ayurveda, Siddha, traditional Chinese, traditional Malay, Unani and other traditional systems of medicine are commonly practised. Therefore, Malaysia is to become a significant global player given that the global trade of herbal products, which amounted to RM777 billion in 2006 and is projected to triple by 2020 (Anonymous, 2011). Malaysia's herbal industry has been identified as one of the agriculture Entry Point Projects (EPPs) under the National Key Economic Areas (NKEAs) in the Economic Transformation Programme (ETP). Several popular Malaysian herbs have been identified as the focus, including *Eurycoma longifolia* (Tongkat Ali), *Labisia pumila* (Kacip Fatimah), *Orthosiphon stamineus* (Misai Kucing), *Andrographis paniculata* (Hempedu Bumi) and *Phyllanthus niruri* (Dukung Anak) (Anonymous, 2011).

Herbal medicine or phytomedicine refers to the use of a plant's seeds, berries, roots, leaves, bark or flowers for medicinal purposes. Historically, herbal drugs were used as tinctures, poultices, powders and teas followed by formulations, and lastly as

pure compounds. There are 119 pure chemical compounds extracted from higher plants used in medicine and 74% of these compounds came from folklore claims (Gurib-Fakim, 2006). WHO recognizes herbal medicines as valuable resources and states it is necessary to develop a systematic inventory of medicinal plants to introduce regulatory measures, apply good manufacturing practices and to include herbal medicines in the conventional pharmacopoeia of each nation (WHO, 1998a).

Well-known examples of plant-derived prescription drugs include the antimalarial drug quinine obtained from the bark of *Cinchona officinalis*; the analgesics, codeine and morphine from *Papaver somniferum*; the antihypertensive reserpine from *Rauwolfia serpentina*; and the cardiac glycoside, digoxin, from *Digitalis purpurea* (Cragg and Newman, 1999). Initially, quinine from cinchona bark was used to manage the symptoms of malaria long before the disease was identified and the raw ingredients of a common aspirin tablet have been a popular painkiller for far longer than we have had the access to tablet-making machinery (Ansari and Inamdar, 2010).

Tropical rain forests continue to support a vast reservoir of potential drug from plant species due to their ability to synthesize various chemicals as defense agents against pests, diseases and predators. They are an excellent reservoir of medicines and chemical leads with which researchers can design and synthesize new drugs (Zakrzewski, 2002). The potential for finding more compounds is enormous as to date only less than 1% of tropical forest plant species have been tested for chemical compounds and medicinal value, yet at least 25 % of all modern drugs originally came from rainforests (Rates, 2001). Due to the disappearance of tropical plant

species, there is a sense of urgency to increase screening efforts and investigate commercial potential of tropical plant chemicals.

2.1.2 Safety concern on the use of pharmaceutical from plants

Safety is a fundamental principle in the provision of herbal medicines and a critical component of quality control. Herbal medicines are generally considered as safe based on their long-standing use in various cultures. However, there were case reports of serious adverse effects after administration of herbal products. High profile issues such as adverse reactions associated with *Ephedra* and *Aristolochia* have shown that herbal medicine can produce toxicity after consumption in humans (Jordan *et al.*, 2010). A number of other plant-derived agents were entered into clinical trials and were terminated due to the lack of efficacy or unacceptable toxicity. Some examples are acronycine, bruceantin, maytansine and thalicarpine (Cragg *et al.*, 1993). Some adverse effects reported in association with herbal products are attributable to quality issue. Toxicity associated with herbal medicines may result from misidentification of plant species, contamination with toxic metals and pesticide, adulteration with pharmacologically active synthetic compounds and herb-drug interactions (Ernst, 2004; Van Breemen *et al*., 2008).

This issue has led to the regulation of traditional medicines through legally enforced product registration in Malaysia in January 1992. However, herbal medicines are not rigorously regulated by the Drug Control Authority (DCA) of Malaysia. The evaluation of the quality and safety of herbal medicines by the DCA is limited to control on the content of specified adulterants and contaminants such as heavy metals and micro-organisms (Aziz, 2009). Given that herbal medicines are often assumed as being harmless, the public may be at risk of adverse effects from

the use of herbal medicines. The scientific evaluation of safety and efficacy of herbal products and medicinal preparation is thus of vital importance from both medicinal and economic perspectives.

2.2 *Euphorbia*

Euphorbia is a genus of flowering plants belonging to the family Euphorbiaceae, comprises about 300 genera and 5,000 species, common to tropical countries (Figure 2.1). The genus *Euphorbia* is the largest in spurge family, comprising more than 2000 species (Aslam *et al*., 2014). It is characterized by the presence of white milky latex which is toxic to a certain extent (Kumar *et al.,* 2010).

2.2.1 *Euphorbia hirta* L.
2.2.1.1 Botanical description

E. hirta is a small annual, branched herb prostrate to ascending with branches reaching 70 cm in height (Figure 2.2). It is reddish or purplish in color, with abundant latex and is covered with short hairs. Its leaves are opposite, distichous and simple (not lobed or divided). The leaf blades are lanceolate-oblong, long elliptic or ovate-lanceolate. The inflorescence of *E. hirta* has a terminal or axillary cluster of flowers, called a 'cyathium', with several cyathia densely clustered into a cyme (Figure 2.3). The flowers of *E. hirta* are unisexual; the male flowers are sessile, the bracteoles are linear, fringed, the perianth is absent and possesses one stamen, whereas the female flowers have short pedicel, the perianth is rimmed, the ovary is superior, covered with short hairs, three-celled, possesses three styles, minute and the apex is two-fid. The fruit of *E. hirta* is exerted, acutely three-lobed, base truncate, covered in short hairs and three-seeded. The seeds are oblong, four-sided

prismatic, slightly wrinkled, pinkish brown and caruncle absent. Flowering duration of individual plant is usually throughout the year. *E. hirta* often grows in cultivated areas in lowland, paddy fields, gardens, roadsides and waste places. They prefer dry condition, from sea-level up to 2000 m altitude. *E. hirta* has many synonyms in different countries. The common names for *E. hirta* are snakeweed, asthmaweed, hairy spurge and milkweed. In Malaysia, it is called ambin jantan, kelusan, gelang susu or keremak susu (Huang *et al.*, 2012). Figure 2.1 shows the taxonomic classification of *E. hirta* plant.

2.2.1.2 Origin and geographical distribution

E. hirta is native to Central America and a very common weed of the tropics and subtropics; it occurs throughout tropical Africa and also in South Africa (PROSEA, 2014)
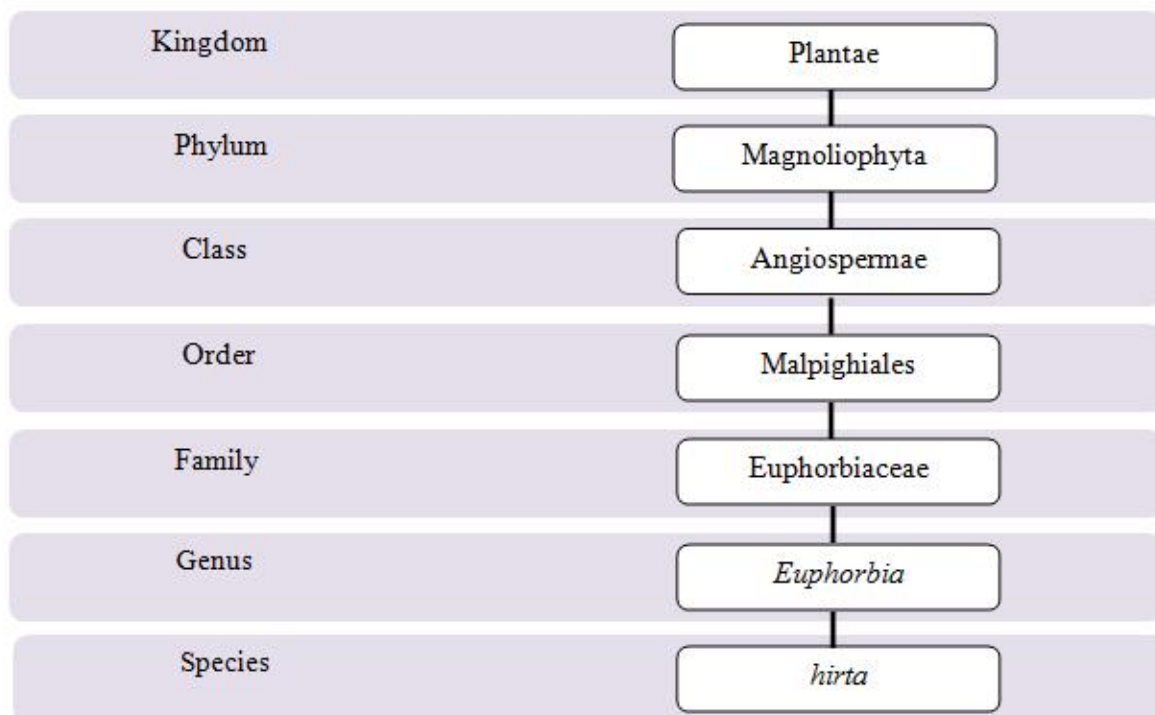
Figure 2.1: Taxonomic classification of *E. hirta* (Mamun-Or-Rashid *et al*., 2013)

Figure 2.2: *Euphorbia hirta* plant
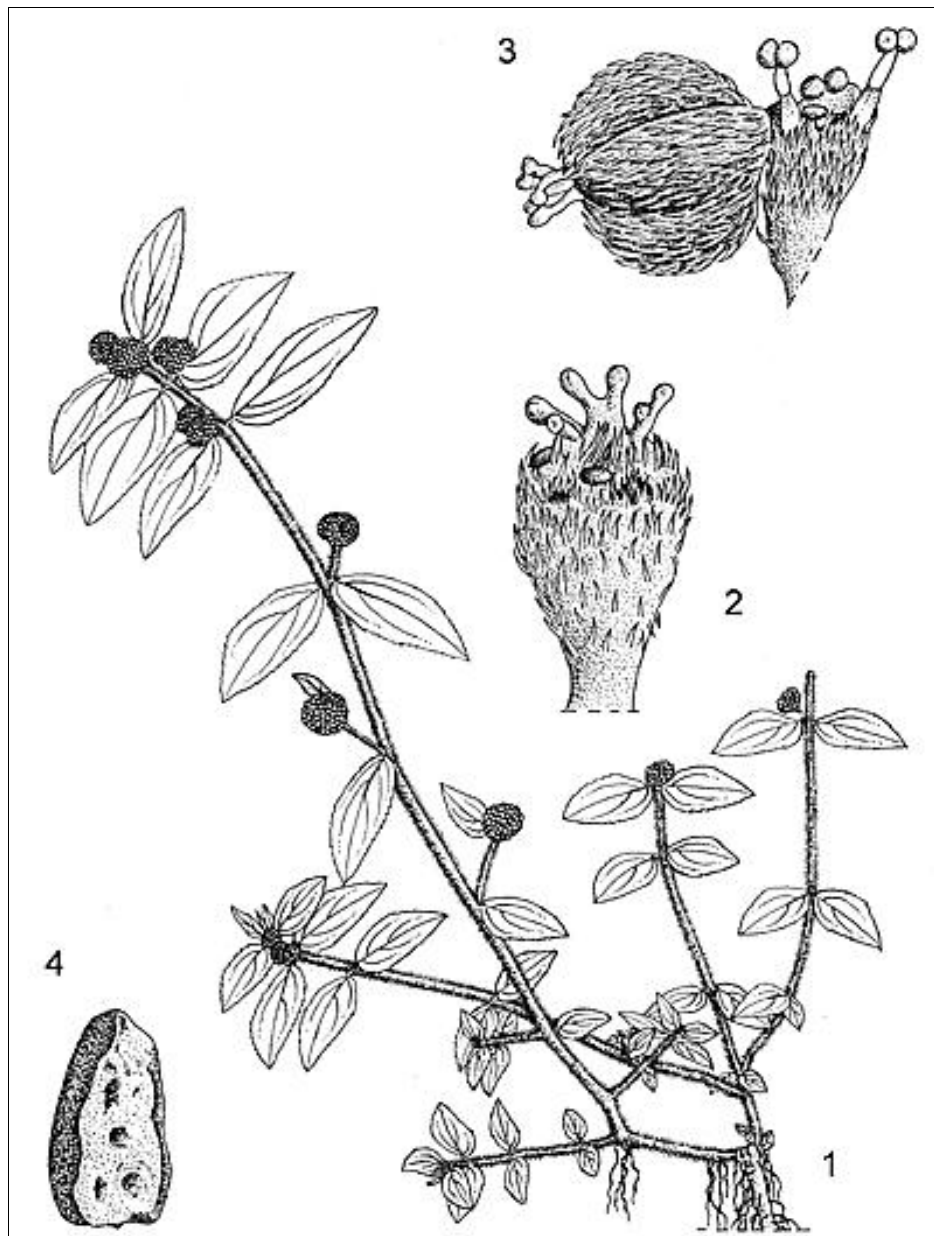
Figure 2.3: *E. hirta* plant (1: plant habit; 2: young cyathium; 3: mature cyathium; 4: seed) (PROSEA, 2014)

2.2.1.3 Phytochemistry

Phytochemical analysis of *E. hirta* showed the presence of reducing sugar, alkaloids, flavonoids, sterols, tannins and triterpenes in the whole plant. Sterols isolated from *E. hirta* are cycloarternol, 24- methylene-cycloarternol, -sitosterol, euphorbol hexacozonate, 1-hexacosanol, tinyaloxin, campesterol and stigmasterol (Shih and Cherng, 2012). *E. hirta* contains three hydrolysable tannins, namely, dimeric hydrolysable tannin, euphorbin E and the dimeric dehydroellagitannins, euphorbin A and euphorbin B (Yoshida *et al.*, 1990). Polyphenols from the leaves of *E. hirta* were also isolated by using physicochemical and spectroscopic methods: gallic acid, 2,4, 6-tri-O-galloyl-D-glucose, quinic acid ester, 1,2,3,4, 6-penta-Ogalloyl- -D-glucose and 3,4-di-O-galloylquinic acid (Chen, 1991). The triterpenes -amyrin, 24-methylencycloartenol and -sitosterol have been identified from *E. hirta* (Martinez *et al.,* 1999). Flavonol glycosides were isolated from methanolic extract *of E. hirta* and chemically characterized as afzelin, quercitrin, and myricitrin (Liu *et al.*, 2007). The chemical structure of 2,4, 6-tri-O-galloyl-D-glucose, -sitosterol, myricitrin, gallic acid and quercitrin found in *E. hirta* are showed in Figure 2.4.
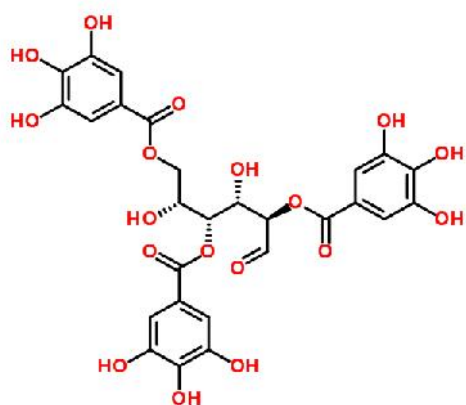
2, 4, 6-tri-O-galloyl-D-glucose   -sitosterol
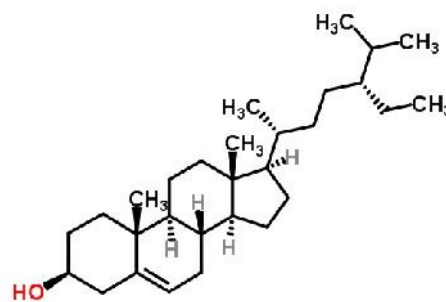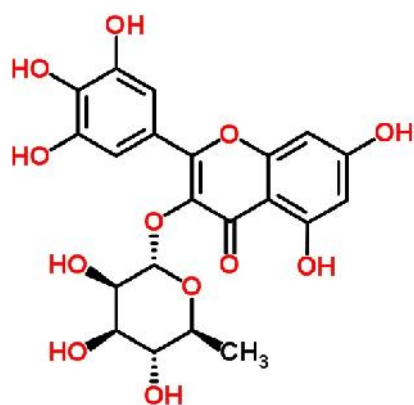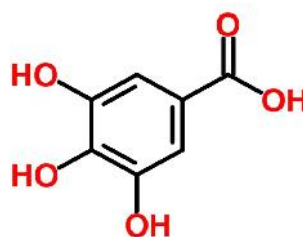
Myricitrin   Gallic acid
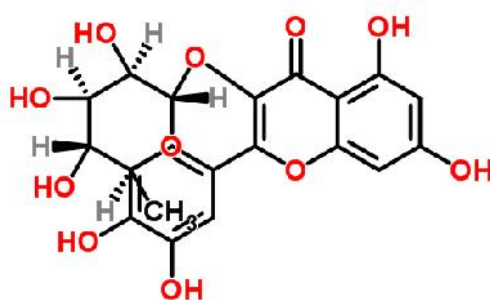
Quercitrin

Figure 2.4: Chemical structure of some known compounds found in *E. hirta*
(Source: http://www.chemspider.com/Chemical-Structure)

2.2.1.4 Pharmacological activities

E. hirta exhibited several pharmacological activities as reported in literature. The antibacterial activity of *E. hirta* has been comprehensively investigated and proven. The ethanolic extract of *E. hirta* inhibited the growth of the *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtili* (Ogbulie *et al.*, 2007). Aqueous and chloroform leaf extracts of *E. hirta* possess an antibacterial activity against *Klebsiella pneumonia* (Suresh *et al*., 2008).

The antidiarrheal effect of the herb decoction was studied in mice. The lyophilised decoction of *E. hirta* was found to possess antidiarrheal activity induced by castor oil, arachidonic acid and prostaglandin E2 (Galvez *et al*., 1993a). Quercitrin isolated from *E. hirta* showed an antidiarrheal activity at a dose of 50 mg/kg against castor oil and prostaglandin E2-induced diarrhea in mice (Galvez *et al*., 1993b).

The antioxidant activity of *E. hirta* was comparable with that of ascorbic acid and found to be dose dependent (Basma *et al*., 2011). The aqueous extract of *E. hirta* showed an antioxidant effect and a free radical scavenging activity in various *in vitro* models like total antioxidant and total ferric reducing power determination, assay for free radical-scavenging activity using ABTS, DPPH and hydroxyl radical scavenging assays. It showed maximum antioxidants and free radical scavenging activities at 0.25 mg/mL (Sharma *et al.,* 2007).

E. hirta was reported to possess antimalarial activity. The bioassay-guided fractionation of methanol extracts of the aerial parts of *E. hirta* resulted in isolation of flavonol glycosides afzelin, quercetrin and myricitrin. The compounds showed

inhibition of the proliferation of *Plasmodium falciparum* with IC$_{50}$ values of 1.1, 4.1, 5.4 µg/mL, repectively. Furthermore, they showed little cytotoxic activity against human epidermoid carcinoma KB3-1 cells (Liu *et al.,* 2007).

Antiinflammatory effects of *E. hirta* were exhibited in 12-o-tetradecanoyl phorbol acetate-induced ear inflammation in mice whereby the result showed a dose-dependent effect (Martinez *et al.*, 1999; Lanhers *et al.*, 1991). In some recent studies, *E. hirta* produced a remarkable antiinflammatory effect via its active component of beta-amyrin and showed a dose-related inhibition against LPS-induced NO production (Camuesco *et al.*, 2004; Shih *et al.*, 2010).

E. hirta was found to have an antiasthmatic activity due to the relaxation effect on the bronchial tubes and a depressant action on respiration (Kumar *et al.*, 2010). *E. hirta* reduced asthma attack has been shown as effective as corticosteroid did in the BALB/c asthmatic mouse mode (Ekpo and Pretorius, 2008). The possible active component of *E. hirta* is thought to be quercitrin. *E. hirta* ethanol extract significantly prevented eosinophil accumulation and eosinophil peroxidase activity and reduced the protein content in bronchoalveolar lavage fluid in a 'mild' model of asthma (Singh *et al.*, 2006).

2.2.1.5 Ethnopharmacology

E. hirta plays a very important role in traditional use due to its wide range of biological and pharmacological properties. The plant part used are the whole plant, latex, aerial part, root and leaf. Different formulations are used, including crude drug, decoction, infusion, lotion and powders. The latex and fluid extract of the tincture are used to treat gastrointestinal disorders (diarrhea, dysentery, intestinal

parasitosis), bronchial and respiratory diseases (asthma, bronchitis, hav fever) (Kumar *et al.*, 2010). The decoction of the root is used to alleviate vomiting. Decoction of dry herbs is used for skin diseases. Root decoction is used for snake bites, sores, wounds, boils and is beneficial for nursing mothers with deficient milk. The entire plant is prescribed as an antidote; it is considered hemostatic, sedative and narcotic. In Australia, the most common use of *E. hirta* is to treat hypertension, asthma, edema and pectoral complaints. In the Philippines, leaves are mixed with datura metel leaves and flowers in the preparation of "asthma-cigarettes" (Huang *et al.*, 2012).

2.2.1.6 Plant derived anticancer agents

Plants have enormous contribution to modern medicine with the origin of numerous drugs in use today are derived from plants. Most of these plant-derived drugs were originally discovered through the study of herbal cures and folk knowledge of traditional people. Some of these could not be synthesised economically and are still obtained from wild or cultivated plants despite the enormous advancement in synthetic chemistry. Natural product secondary metabolites from plants and microbes in particular play a very important role in the amelioration of cancer. In the field of anticancer activity, a correlation between biological activity and plants used in folklore has been validated (Farnsworth and Kaas, 1981). More than 60% of all cancer drugs are of natural origin and a majority of these compounds are obtained from higher plants (Newmann *et al.*, 2003).

In the 1970s, one of the significant breakthroughs in the field of anti-cancer drugs comes from the Madagascan Periwinkle. The Periwinkle has a long history of treating a wide variety of diseases and has also been used for centuries against

diabetes. This traditional use as a cure for diabetes resulted in preliminary laboratory investigations. Laboratory animals developed significantly low counts of white blood cells leaving them defenseless against infections caused by bacteria. The result showed that one or more of the Periwinkle alkaloids might slow or halt white blood cell production and this is probably the mechanism that evolved in nature to discourage herbivorous predators from eating the Madagascan Periwinkle. Hence, further bioassay-guided isolation of the plant led to the characterization or the active complex alkaloidal compounds namely vincristine and vinblastine. The level of vincristine in the plant is extremely low (0.0002%) and thus making it a very expensive antitumour agent. They have been proven to be effective agents against childhood leukaemia, breast cancer and Hodgkin's disease. Vincristine and vinblastine exert their anticancer properties by inhibiting mitosis by binding to tubulin, thus preventing the cell from making spindles it needs to be able to move its chromosomes around as it divides (Gurib-Fakim, 2006).

Other examples of plant-derived anticancer drug are etoposide and teniposide, which are semisynthetic derivatives of the natural product epipodophyllotoxin. Epipodophyllotoxin is an isomer of podophyllotoxin that was isolated as the active antitumor agent from the roots of various species of the genus *Podophyllum*. These plants have a long history of medicinal use by early American and Asian cultures, including the treatment of skin cancers and warts. Etoposide is now marketed as Vepesid for small cell lung cancer, testicular cancer and lymphomas while Teniposide is used in treating brain tumours (Cragg and Newman, 1999). Recent plant-derived anticancer drug are homoharringtonine isolated from the Chinese tree, *Cephalotaxus harringtonia* (Cephalotaxaceae) and elliptinium, a derivative of

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ellipticine isolated from species of plant family Apocynaceae including *Bleekeria vitensis*, a Fijian medicinal plant with reputed anti-cancer properties. Combretastatin isolated from the bark of the South African tree *Combretum caffrum* Kuntze is active against colon, lung and leukemia cancers. It is considered to be the most cytotoxic phytomolecule isolated so far (Unnati *et al.,* 2013).

2.3 Cell death

Cell death is a general biological phenomenon, which continues throughout the lives of organisms and is important to maintain normal cellular homeostasis. Cell deaths during embryonic development are vital for successful organogenesis and the crafting of complex multicellular tissues (Nika and Stanley, 2004). Defects in apoptotic cell death regulation may lead to many diseases. Acquired Immunodeficiency Syndrome (AIDS), neurodegenerative disorders, insulin-dependent diabetes, hepatitis C infection, myocardial infarct and atherosclerosis are examples of disorders with inappropriate apoptosis or increased rate of apoptosis, whereas autoimmune diseases and cancer are disorders with decreased rate of apoptosis (Ulukaya *et al.*, 2011).

During cell death, activation of distinct biochemical cascades follows and manifest with different morphological features. Cell death can occur by either of two distinct mechanisms, namely, apoptosis or necrosis. Apoptosis or programmed cell death is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. The biochemical and morphological events that lead to apoptosis usually have a highly regulated series of events. Necrosis or accidental cell death is a pathological process

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in response to trauma generated by external factors or overwhelming cellular injury (Figure 2.5) (Fa´tima and Pedro, 2010).

2.3.1 Apoptosis and necrosis

The term 'apoptosis' was first coined by Kerr *et al*. (1972) to describe the specific morphological features leading to the programmed cell death. Apoptosis is of Greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers (Sankari *et al*., 2012). This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms.

Apoptosis is a form of cell death characterized by cell shrinkage, membrane blebbing, chromatin condensation and looses contact to its neighbouring cells. Apoptotic cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin and organelles. Apoptosis is an active process as the apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. These morphological changes are a result of characteristic molecular and biochemical events leading to the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal and cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste and Pulkki, 2000).

The term necrosis was originally used to define all types of cell death until Kerr (1971); Kerr *et al.* (1972) introduced the concept of apoptosis. Necrosis is of Greek origin, having the meaning "corpse" (Zong and Thompson, 2006). For a long

time, necrosis has been considered an accidental and uncontrolled form of cell death lacking underlying signaling events. Morphologically, necrosis is characterized by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and loss of intracellular contents (Duprez *et al.*, 2009). During necrosis, the intracellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory reaction in the corresponding tissue (Leist and Jaattela, 2001).
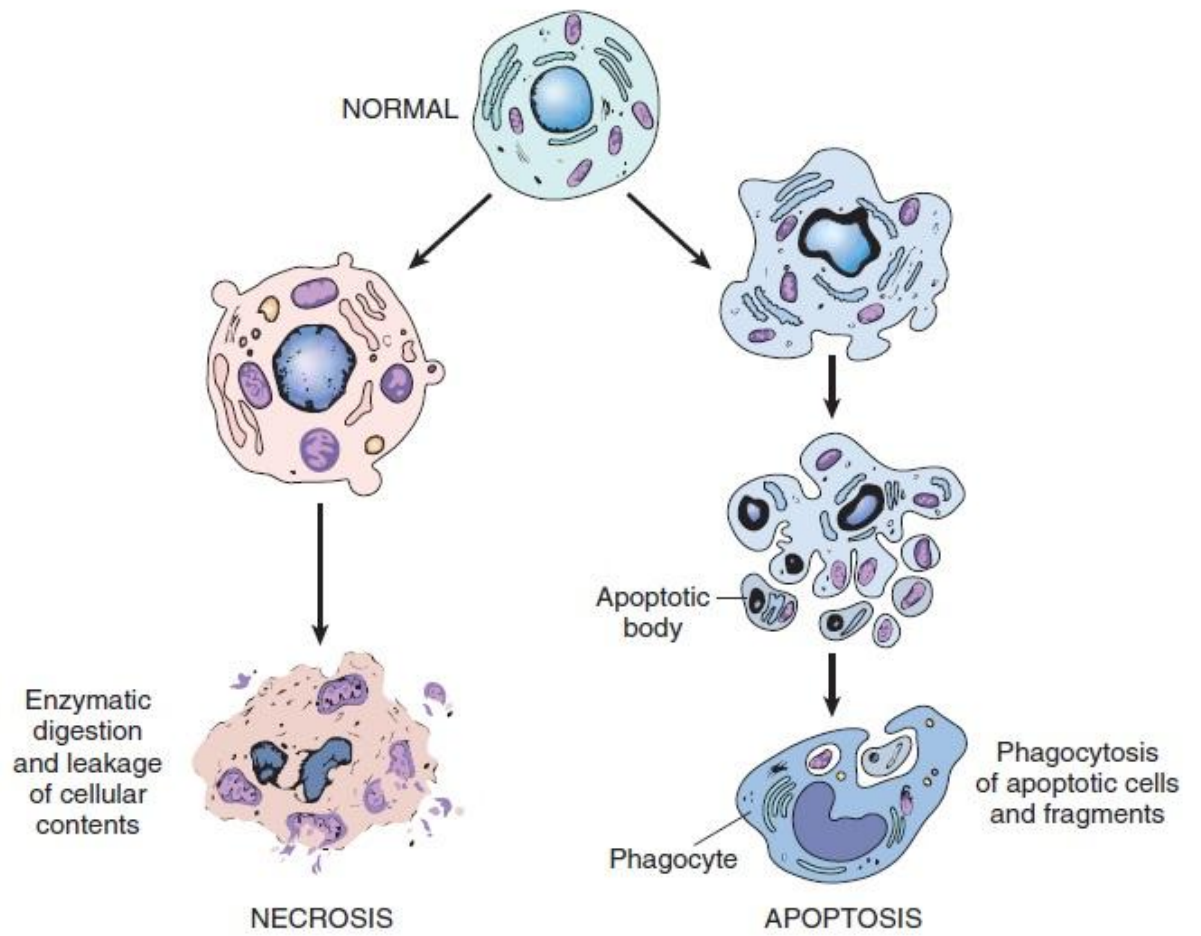
Figure 2.5: Types of cell death [Source: Walker *et al.*, (1988)].

2.3.2 Mechanism of apoptotic and necrotic cell death

2.3.2.1 Apoptosis pathways

Occurrence of apoptosis is controlled by a number of complex proteins, which are activated by various triggers and arranged in sequential signaling modules. There are two main pathways that lead to apoptosis. The first, referred to as the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal. The second pathway is the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily. Both pathways converge to a final common pathway involving the activation of caspases that cleave regulatory and structural molecules resulting in the death of the cell (Ghobrial *et al.*, 2005).

2.3.2.1.a Intrinsic pathway

In the intrinsic or mitochondrial pathway of apoptosis, caspase activation is closely linked to permeabilization of the outer mitochondrial membrane (Green and Kroemer, 2004). This permeabilization is regulated by proteins from the Bcl-2 family. The Bcl-2 family includes proapoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, and antiapoptotic members such Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1. Antiapoptotic Bcl-2 members act as supressors of apoptosis by blocking the release of cytochrome-c, whereas proapoptotic members act as promoters (Reed, 1997).

Upon triggered by a death signal, proapoptotic proteins undergo posttranslational modifications that include dephosphorylation and cleavage resulting in their activation and translocation to the mitochondria leading to apoptosis. The activation of Bcl-2 members such as Bax and Bak may cause an increase of mitochondrial membrane permeability, leading to the release of cytochrome-c and second mitochondria-derived activator of caspase (SMAC) or inihibitor of apoptosis proteins (IAPs) into cytosol. The release of cytochrome c then binds and activate with Apaf-1, as well as procaspase-9, forming an "apoptosome". Activation of caspase-9 then activates caspase-3, which subsequently activates the rest of the downstream caspases and leads to apoptosis (Ghobrial *et al.*, 2005).

2.3.2.1.b Extrinsic pathway

The extrinsic pathway or death receptor pathway initiates apoptosis *via* transmembrane receptor-mediated interactions. These involve death receptors that are members of the TNF receptor gene superfamily (Susan, 2007). These proteins comprise death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain, and caspases 8 and 10 (Ghobrial *et al.*, 2005). Activation of the extrinsic pathway is initiated with the ligation of cell surface receptors called death receptors (DRs). Fas is a member of the tumor necrosis factor receptor superfamily, also known as Apo-1 or CD95. Other TNF receptors include TNF R1, DR3 (Apo 2), DR4 (TRAIL R1), DR5 (TRAIL R2), and DR6 (Zapata *et al.*, 2001).

Following a death stimulus, the FasL interacts with the inactive Fas complexes and forms the death-inducing signaling complex which contains the adaptor protein Fas-associated death domain protein and caspases 8 and 10. This