PHYTOCHEMICAL CHARACTERIZATION AND WOUND HEALING PROPERTY OF *Euphorbia hirta* L.

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by

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
cm	Centimeter
CO_2	Carbon dioxide
g	Gram
mg GAE/g	Milligram gallic acid equivalent
mg/mL	Milligrams per milliliter
α	Alpha
β	Beta
δ	Chemical shift
μ	Micro
μL	Microlitre

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AR	Analytical grade
ATCC	American type culture collection
CHC_{l3}	Chloroform
DCM	Dichloromethane
Dd	Doublet of doublet
DMEM	Dulbecco`s modified Eagle medium
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1- picrylhydrazyl
EtOAc	Ethyl acetate
FBS	Fetal bovine serum
FTIR	Fourier Transform Infrared spectroscopy
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HMBC	Heteronuclear multiple bond coherence
HPLC	High Performance Liquid Chromatography
Hs27	Human foreskin fibroblast
HSQC	Heteronuclear Single Bond Coherence
IC_{50}	Concentration of a test substance required for 50% inhibition *in vitro*
MeOH	Methanol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
OD	Optical density

PBS	Phosphate buffer saline
Rf	Retention factor
Rpm	Revolution per minute
s	Singlet
S.E.M	Standard error mean
TLC	Thin layer chromatography
TPC	Total phenolic content
TFC	Total flavonoid content
UV	Ultraviolet
WHO	World Health Organization
wt	Weight

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PENCIRIAN FITOKIMIA DAN SIFAT PENYEMBUHAN LUKA

***Euphorbia hirta* L.**

ABSTRAK

Euphorbia hirta L., yang tergolong dalam keluarga Euphorbiaceae, merupakan tumbuhan semusim yang digunakan secara meluas dalam perubatan tradisional untuk rawatan pelbagai jenis penyakit. Walau bagaimanapun kajian tentang keberkesanan *E. hirta* L dalam penyembuhan luka, terutamanya sebatian bioaktif tumbuhan ini dan mekanisme tindakan dalam penyembuhan luka adalah masih kurang. Matlamat kajian ini memfokuskan pengasingan sebatian aktif utama daripada *E. hirta* yang terlibat dalam aktiviti penyembuhan luka. Bahagian aerial *E. hirta* telah dikutip di bahagian utara Malaysia dan di Iraq, seterusnya diekstrak dengan pelarut yang berbeza kepolaran, iaitu heksana, kloroform, metanol dan air. Kesemua ekstrak disaring untuk kandungan fenolik total dan flavonoid, serta aktiviti antioksidan dan antiinflammatori. Seterusnya, potensi aktiviti penyembuhan luka bagi kesemua ekstrak ini dikaji dengan mengukur migrasi dan kebolehhidupan sel-sel fibroblast manusia, Hs27. Seterusnya, ekstraksi lanjut dilakukan ke atas ekstrak metanol bagi mengenal pasti sebatian aktif. Sebatian yang telah diasingkan dipastikan strukturnya berdasarkan spektroskopi kromatografi jisim cecair (LC-MS/MS), nuklear magnetik resonan (NMR) dan kromatografi cecair prestasi tinggi (HPLC). Selepas itu, pengedokan molekul dilakukan pada sebatian terpencil untuk menganalisis interaksi pengikatannya dengan protein-protein Glycogen Synthase Kinase-3β (GSK3B) dan Casein kinase 1 (CK1). Paras ekspresi mRNA bagi empat sitokin (IL1B, IL6, TNF dan COL1A1) selepas sel fibroblast manusia dirawat dengan ekstrak turut diukur. Ekstrak *E. hirta* menghasilkan beberapa sebatian berbeza: kaempferol, quercetin, astragalin, isoquercitrin, dan β-

sitosterol. Antara kesemua sebatian yang diuji, β- sitosterol menunjukkan peratusan migrasi tertinggi. Pengedokan molekul mendedahkan bahawa semua sebatian terpencil, terutamanya sebatian β-sitosterol, menunjukkan interaksi yang ketara dengan protein GSK3B dan CK1 berbanding dengan drug standard nitrofurazone. Keputusan ini menunjukkan korelasi yang positif antara aktiviti antioksidan sebatian terasing, yang ditentukan oleh enzim antioksida seperti SOD, GPx dan katalase, dan ekstrak metanol dengan aktiviti penyembuhan luka sebatian dan ekstrak tersebut. Sebatian yang diasingkan mempamerkan kesan signifikan dalam ekspresi mRNA empat sitokin yang diukur. Astragalin dan kaempferol menunjukkan pengawalaturan menurun ekspresi IL1B dan IL6, manakala ekstrak metanol menunjukkan pengurangan ketara TNF dan peningkatan ekspresi COL1A1. Kesimpulannya, sebatian terasing dan ekstrak metanol *E. hirta* mempamerkan aktiviti penyembuhan luka yang kukuh, dan seterusnya menyokong penggunaan etnoperubatan tumbuhan ini dalam penyembuhan luka.

PHYTOCHEMICAL CHARACTERIZATION AND WOUND HEALING PROPERTY OF *Euphorbia hirta* L.

ABSTRACT

Euphorbia hirta L, an annual plant that belongs to the Euphorbiaceae family, is a common traditional remedy for several diseases. Currently, there are few investigations on this plant as a treatment for wound healing, particularly the bioactive compounds and mechanism of action. Therefore, this study focused on isolating the main active compounds associated with the wound-healing activity of *E. hirta*. The aerial parts of the plant were collected from the northern part of Malaysia and Iraq and then were extracted with solvents of different polarities: hexane, chloroform, methanol, and water. All extracts were screened for total phenolic and flavonoid contents and antioxidant and anti-inflammatory activities. Additionally, all extracts were assessed for potential wound healing activity by measuring the migration and cell viability of human fibroblast cells, Hs27. Then the methanol extracts were further fractionated to identify the active compounds. The structure elucidation of isolated compounds was confirmed using liquid chromatography-mass spectrometry (LC-MS/MS), nuclear magnetic resonance (NMR), and high-performance liquid chromatography (HPLC). Subsequently, molecular docking was performed on the isolated compounds to analyse their binding interactions with the Glycogen Synthase Kinase-3β (GSK3B) and Casein kinase 1 (CK1) proteins. The level of mRNA expression of four cytokines (IL1B, IL6, TNF, and COL1A1) after treating human fibroblasts with different compounds were assayed. The *E. hirta* extract yielded a different type of compounds: kaempferol, quercetin, astragalin, isoquercitrin, β-sitosterol. Among all the tested compounds, β-sitosterol revealed the highest migration

percentage. The molecular docking revealed that all isolated compounds showed significant interactions with the GSK3B and CK1 proteins compared to the standard drug nitrofurazone, particularly β-sitosterol. The results showed a positive correlation between the antioxidant activity, which was determined by antioxidant enzymes such as SOD, GPx, and catalase, of the isolated compounds and methanolic extract with their wound healing activity. The isolated compound showed a significant effect of the mRNA expression of the four cytokines. Astragalin and Kaempferol showed significant downregulation of IL1B and IL6 expression, while methanolic extract showed significant downregulation of TNF and upregulation of COL1A1 expression. In conclusion, the isolated compounds and *E. hirta* methanolic extract demonstrated substantial wound healing activities, thus supporting the ethnomedicinal usage of this plant in wound healing.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Herbal medicine has a long history across human civilization (Megh Goyal, 2018). According to the World Health Organization (WHO), more than 80% of the population in underdeveloped countries utilize traditional medicine to maintain good health, of which approximately 85% originate from plants (Muskiewicz, 2017). Furthermore, 35% of medicine was sourced from natural products (Calixto, 2019).

A wound can be defined as a physical, chemical, or thermal injury that results in an opening or breaking in the integrity of the skin (Shenoy et al., 2009). A wound also denotes a breakdown in the skin's epithelial integrity, which may affect the underlying normal tissue's structure and function. (Dewangan et al., 2012; McGready et al., 2001). The wound ranges from a simple break in the epithelial integrity of the skin and could extend into the subcutaneous tissue. In addition, a wound might destroy other structures, such as bone Fields, tendons, arteries, nerves, muscles, and parenchymal organs (Velnar et al., 2009). Wounds are categorized as acute and chronic depending on the healing period (Majumdar et al., 2007; Manuskiatti & Maibach, 1996). Wounds that are repaired in a well-organized pathway from 5 to 10 days or within 30 days are known as acute wounds. In contrast, chronic wounds are skin injuries that do not undergo the normal stages of healing due to various factors, such as infection, necrosis, exudate, tissue hypoxia, and superfluous levels of inflammatory cytokines, that can prolong one or more stages of healing (Velnar et al., 2009). Normal wound healing is divided into four stages in the following order (Guo & DiPietro, 2010): coagulation and haemostasis, inflammation, proliferation, and remodeling

(Brown, 2015; Velnar et al., 2009). Several factors can delay or interrupt one or more phases of this process and impair wound healing (Brown, 2015; Dryden et al., 2013), including improper diet, infection at the wound site, drugs, old age, diabetes, and other diseases (Guo & DiPietro, 2010).

Herbal medicines demonstrate potential in wound healing; the natural compounds in the herbs may promote wound healing and tissue regeneration through varied mechanisms (Schmidt et al., 2009). For instance, *Euphorbia hirta* L. ((Euphorbiaceae) is an annual plant that grows wild in tropical and subtropical countries and possesses anti-inflammatory and wound-healing effects due to the flavonoid presence in the whole plant extract (Meher et al., 2012; Rana et al., 2019). This species, which has long been used as a traditional remedy for respiratory ailments (asthma, cough, bronchitis, hay fever) and gastrointestinal illnesses (diarrhea, dysentery, intestinal parasitosis, etc.), has garnered attention in recent studies (Salehi et al., 2019). The pharmacological activities of *E. hirta* include antioxidant, antimicrobial, antiepileptic, sedative anxiolytic, anti-inflammatory, pain and fever relief, antihistaminic, antidiabetic, anticancer, gastrointestinal, wound healing, diuretic, antiparasitic, immunological, liver protection, galactogenic, angiotensin-converting enzyme inhibitor and anti-dipsogenic (Al-Snafi, 2017; Kumar et al., 2010).

1.2 Problem Statement

Wounds are a developing danger that impacts a large portion of the population and represent a significant global burden on public healthcare. (Mayya et al., 2021). It is estimated that 1 to 2 percent of the population in developed countries will suffer from a chronic wound in their lifetime. In the United States, it is reported that chronic wounds affect approximately 6.5 million patients. Wound healing successfully

proceeds through the clearly defined sequence of the individual phases described above, coagulation and haemostasis, inflammation, proliferation, and remodeling. Chronic (non-healing) wounds/ulcers fail to complete some individual stages and the entire healing process and stagnate usually at the early inflammatory stage. Chronic wounds are defined as those that do not follow the normal healing process and show no signs of effective healing within 3 months after the tissue injury. The features characteristic for chronic wounds are prolonged or excessive inflammatory phase, overabundant neutrophil infiltration, persistent infections, and frequent formation of tissue/organ atypical biofilms. Under normal conditions, healthy persons rarely experience issues with wound healing, which often follows the reparative process in a timely and organized manner. However, the wound healing process may be impaired by a variety of physio-pathological circumstances, including stress, obesity, diabetes, hypoxia, age, malnutrition, and immunodeficiency (Mayya et al., 2021). The impact of chronic wounds on the health and quality of life of patients and their families should not be underestimated. Patients with chronic wounds may experience chronic pain, loss of function and mobility, increased social stress and isolation, depression and anxiety, prolonged hospitalization, increased financial burden, and increased morbidity and mortality. The incidence of chronic wounds is expected to increase as our population ages. In the USA alone, over 25 billion US $ are spent annually for the treatment of chronic wounds affecting around 6.5 million patients. Hence, the benefits of moist wound healing are up to 50% faster wound healing, increased re-epithelialization, lower rate of infection, less scarring and better cosmetic results, prevention of bacterial strike through from outside the wound to the wound surface, shortening of the inflammatory and proliferative phases of wound healing with more rapid progression into the remodeling phase (Brown, 2015; Velnar et al., 2009).

Throughout history, medicinal plants have gained widespread acceptance due to their lower side effects compared to synthetic medicine. Nowadays, the utilization of medicinal plants in the treatment of various diseases is increasing. Furthermore, phytomedicines have served as the primary starting point for the discovery and development of new medications based on the traditional use of plants. Honey, curcumin, garlic, and aloe vera are among medicinal plants-derived natural products that are scientifically proven to exhibit wound healing activity. (Saikaly & Khachemoune, 2017; A. Sharma et al., 2021). Improving natural products' effects can provide novel wound-healing therapies for those who trust traditional compounds over synthetic drugs to reduce medical inequalities.

E. hirta plant which widely grows in Malaysia, has several pharmacological studies like antioxidant, antimicrobial, and anti-inflammatory (Al-Snafi, 2017; Kumar et al., 2010). Despite a few studies on *E. hirta* plant extracts for the treatment of wound healing, there are still no studies to investigate their bioactive compounds for wound healing and no scientific report confirming the effectiveness of these compounds on the healing process. At the same time, the current study was designed to investigate the potential bioactive phytochemicals of *E. hirta* plant through *in vitro* and *in silico* studies.

1.3 Research Objectives

This study aimed to evaluate the wound healing property of *E. hirta* plant and to characterize the phytochemical contents that may be responsible for such activity *in vitro* and *in silico.*

Furthermore, the objectives of this study were elaborated as follows:

1. To determine in vitro antioxidant, and wound healing activities of various *E. hirta* extracts.
2. To characterize the phytochemical components of *E. hirta* by using several tools such as qualitative analysis using HPLC, LCMS and GCMS as well as isolation and structure elucidation of secondary metabolites.
3. To evaluate the *in vitro* and *in silico* wound healing properties of the identified compounds from *E. hirta*.

1.4 Significance of the Study

This thesis is working on the investigation of the effects *E. hirta* plant extracts and their isolates on wound healing. This study outcomes would provide a better understanding on the role of *E. hirta* and its phytochemicals for treating wounds through *in vitro* and *in vivo* models. Apart from that, this study would develop chromatographic methods to qualitatively and quantitatively analyse the extracts of *E. hirta*. The outcomes of this study would confirm the wound healing property of *E. hirta* and provide phytochemical information that would benefit the academic community, the herbal industry, and the authority to ensure the quality and safety of the traditional medicine until it is consumed by the patient or customer.

CHAPTER 2

LITERATURE REVIEW

2.1 Wound and the Healing Process

Wound is defined as physical, chemical or thermal injuries that cause an opening or breaking in the integrity of the skin (Shenoy et al., 2009). Another definition, wound is a breakdown in the epithelial integrity of the skin that could cause a disturbance in the structure and function of underlying normal tissue (Dewangan et al., 2012; McGready et al., 2001). It ranges from a simple break in the epithelial integrity of the skin to deeper extending into subcutaneous tissue. It might include destruction to other structures such as tendons, vessels, nerves, muscles, parenchymal organs and bone (Velnar et al., 2009).

Regarding to their healing time frame, wounds can be characterized as acute or chronic (Majumdar et al., 2007; Manuskiatti & Maibach, 1996). Wounds that ordinarily repair themselves with time and in well-organized pathway are categorized as acute wounds. Commonly the time of healing ranges from 5 to 10 days or within 30 days. Meanwhile Chronic wound can be categorized as the skin injury that are unable to heal successfully through the normal stages of healing. The healing stage of chronic wound is troubled by different factors that can prolong one or more stages of healing, like infection, necrosis, exudate, tissue hypoxia, and superfluous levels of inflammatory cytokines (Velnar et al., 2009). The process of normal wound healing can be categorized into four stages (Guo & DiPietro, 2010) which consist of coagulation and haemostasis, inflammatory, proliferation, and remodeling phase (Brown, 2015; Velnar et al., 2009) . Many factors can delay or interrupted one or more phases of this process which causing impaired wound healing (Brown, 2015; Dryden

et al., 2013). Some of these factors are improper diet, infection at the wound site, drugs, elderly age, diabetes and other disease conditions (Guo & DiPietro, 2010)

2.1.1 Wound Healing Phases:

The basic concepts of wound healing can be understood by knowing the physiology of normal wound healing, as well as the phases of haemostasis, inflammation, granulation, and maturation (Mani, 2014).

2.1.1(a) Phase 1: Hemostasis Phase

Hemostasis is the first phase of wound healing. It initiates at the time of injury and has the primary goal of ending bleeding. During this phase, the body's emergency repair system, the blood clotting system, and forms a barrier to block the drainage(Dogan, 2019).

2.1.1(b) Phase 2: Inflammatory Phase

Inflammation is the second stage of wound healing. It initiates soon after the injury, when the wounded blood vessels release transudate (a mixture of water, salt, and protein), that can cause localized swelling. The primary goal of the inflammation phase is to kill bacteria and remove debris, effectively preparing the wound bed for the creation of new tissue. The population of these cells' peaks between 1- 2 days after the damage, and then rapidly drops off after three days. This phase usually lasts 4 to 6 days and is characterized by edema, erythema (skin reddening), heat, and pain (Dogan, 2019).

2.1.1(c) Phase 3: Proliferative Phase

After the wound is cleaned out, it enters the proliferative phase. The goal of this phase is to fill and cover the wound. There are three main stages in the proliferative phase: Filling the wound, contracting the wound edges, and covering the wound (epithelialization). The proliferative phase might last anywhere between four and twenty-four days (Dogan, 2019).

2.1.1(d) Phase 4: Maturation Phase

This stage is the final step of healing. At this phase, the new tissue gradually increases strength and flexibility. Collagen fibers restructure, the tissue remodels and matures, and the tensile strength of the tissue increases (though maximum strength is limited to 80% of the pre-injured strength). Maturation varies significantly from wound to wound, and it might take from 21 days to two years (Dogan, 2019).

2.2 The plant of interest: *Euphorbia hirta*

2.2.1 Phytochemical and Wound Healing Properties of Genus *Euphorbia*

Euphorbiaceae is one of the major families in the plant kingdom covering up to 300 genera and 7,500 species. It is usually found as monoecious herbs, shrubs, and trees that characterized by its latex (milky liquid coming from the trees). Among all genera of Euphorbiaceae, *Euphorbia* is the largest genus, consisting of about 2000 species.

The family of Euphorbiaceae is well known a for its secondary metabolite diversity of isoprenoid biosynthetic pathway. Specifically, *Euphorbia* is reported to have rich in phloracetophenones, cerebrosides, glycerols, sesquiterpenoids, steroids,

and flavonoids. (Goyal, Nagori, & Sasmal, 2012). Al-Snafi (2017) reported the presence of tannins (euphorbins), triterpenoids (lupeol, lupeol acetate, β-amyrin and betulin) and phytosterols (β-sitosterol) in plants of *Euphorbia* genus. Flavonoids (kaempferol, myricetin, rutin, quercetin, and their derivatives) and phenolic compounds (Noori et al., 2009), along with the volatile components such as α terpineol, α-humulene, linalool, β-caryophyllene, terpinene, and germacrene-D (Fokialakis et al., 2003) were also reported from this genus. The diterpenoids in *Euphorbia* are characterized by their skeletons such as jatrophane, lathyrane, myrsinane, daphnane, ingenane, tigliane, pepluane, paraliane, and segetane (Jassbi 2006; Sulyok et al., 2011).

Some species of the genus *Euphorbia* are used in folkloric medicines to cure skin disorders, gonorrhea, intestinal parasites, migraine, and wart (Ertas et al., 2015). The latex of these plants contains numerous natural compounds, some of which are of therapeutic importance or commercial use (Giner and Schroeder, 2015). Few *Euphorbia* species have also been reported for wound healing potential and the phytoconstituents in *Euphorbia* species has support these activity (Goyal et al., 2012).

Some of the secondary metabolites (Table 2.1) presence in *Euphorbia* plants such as tannins, polysaccharides, flavonoids, and sterols, have been reported to have wound healing activity (Ghosh and Gaba 2013). The presence of these phytochemicals may be responsible for the topical applications of some plants to treat wound (Deriso & Hsu, 2010). These phytochemicals help in one or more phases of wound healing process in a positive manner in proper sequence. Based on Table 2.1, there are few other pharmacological activities that also play important role in process of wound healing. They are antioxidant, anti-inflammatory, antimicrobial and analgesic activities.

Table 2.1 Phytoconstituents from *Euphorbia* species and their role in wound healing

Phytoconstituents	Effect	Species	References
Tannins	Promote wound healing due to their astringent property; they reduced scar tissue formation by inhibition of the formation and removal of reactive oxygen substances. Additional advantages of tannins include relief of pain, limitation of secondary infection, prevention of loss of plasma and promotion of prolific epithelialization	*Euphorbia heterophylla*	(James, Omale, & Friday, 2010)
Flavonoids	Exert potential wound healing effect by virtue of their antimicrobial, antioxidant, and anti-inflammatory property.	*Euphorbia characias*	(Ozbilgin et al., 2018)
		Euphorbia hirta	(Bigoniya, Agrawal, & Verma, 2013)
Sterols	Promote epithelialization; they have free radical scavenging and antioxidant activities	*Euphorbia hirta*	(Bigoniya et al., 2013)
Saponins	Antioxidant and antimicrobial activity	*Euphorbia neriifolia*	(Bigoniya & Rana, 2007)
Triterpenoids	Astringent and antimicrobial activity	*Euphorbia hirta*	(Al-Snafi, 2017)

Many of herbal plants containing anti-inflammatory, antioxidant, antimicrobial and compounds have been scientifically shown to possess wound healing activity such as tannins, coumarin, etc. (Patel et al., 2011). Furthermore, herbal preparations that possess analgesic and anti-inflammatory activities can be active for pain management of wounds (McGuire et al., 2006). Table 2.2 show the *Euphorbia* species plants with their pharmacological activities that support the wound healing.

Table 2.2 Reported species of *Euphorbia* with anti-inflammatory, antioxidant, analgesic, and antimicrobial activities.

Activity	**Plant name**	**References**
Anti-inflammatory	*Euphorbia hirta*	(Al-Snafi, 2017; Sharma et al., 2014)
	Euphorbia aegyptiaca	(Abo-Dola & Lutfi, 2016)
	Euphorbia dracunculoides	(Majid et al., 2015)
	Euphorbia retusa	(Sdayria et al., 2018)
	Euphorbia acaulis	(Singh et al., 1984)
	Euphorbia nicaeensis	(Cateni et al., 2004)
	Euphorbia Antiquorum	(Saikia, 2016)
	Euphorbia helioscopia	(Park et al., 2001)
Antioxidant	*Euphorbia hirta*	(Al-Snafi, 2017; Sharma et al., 2014)
	Euphorbia dracunculoides	(Majid et al., 2015)
	Euphorbia retusa	(Sdayria et al., 2018)
	Euphorbia golondrina	(Lawrence Monah et al., 2016)
	Euphorbia mili	(Besagas & Gapuz, 2018)
	Euphorbia trigonaa	(Besagas & Gapuz, 2018)
	Euphorbia antiquorum	(Besagas & Gapuz, 2018; Saikia, 2016)
	Euphorbia helioscopia	(Maoulainine et al., 2012; Uzair et al., 2009)
	Euphorbia tirucalli	(Munro et al., 2015)
	Euphorbia tithymaloides	(Saikia, 2016)
	Euphorbia wallichii	(Tantary et al., 2016)
	Euphorbia cyparissias L	(Stankovic & Zlatic, 2014)
Analgesic	*Euphorbia dracunculoides*	(Majid et al., 2015)
	Euphorbia retusa	(Sdayria et al., 2018)
	Euphorbia hirta	(Al-Snafi, 2017)
	Euphorbia prostrata	(Kiprop, 2015)
	Euphorbia royleana	(Bani et al., 1997)
Antimicrobial	*Euphorbia golondrina*	(Lawrence Monah et al., 2016)
	Euphorbia Antiquorum	(Saikia, 2016)
	Euphorbia helioscopia	(Uzair et al., 2009)
	Euphorbia hirta	(Al-Snafi, 2017)
	Euphorbia aleppica	(Kirbag et al., 2013)
	Euphorbia szovitsii	(Kirbag et al., 2013)
	Euphorbia falcata L	(Kirbag et al., 2013)
	Euphorbia denticulata	(Kirbag et al., 2013)
	Euphorbiamacroclada	(Kirbag et al., 2013)
	Euphorbia cheiradenia	(Kirbag et al., 2013)
	Euphorbia virgata	(Kirbag et al., 2013)
	Euphorbia petiolata	(Kirbag et al., 2013)

2.2.2 *Euphorbia hirta*

2.2.2(a) Botanical Information

The taxonomic classification of *Euphorbia hirta* (L.). (Al-Snafi, 2017; Asha, 2014)

Domain:	Eukaryota
Kingdom:	Plantae-- Plants
Subkingdom:	Viridaeplantae
Phylum:	Magnoliophyta, Flowering Plants
Subphylum:	Euphyllophytina, Seed Plants
Infraphylum:	Radiatopses
Class:	Magnoliopsida, Dicotyledons
Subclass:	Dilleniidae
Superorder:	Euphorbianae
Order:	Malpighiales
Family:	Euphorbiaceae, Spurge Family
Genus:	*Euphorbia*
Specific:	*hirta* - L.
Botanical name:	*Euphorbia hirta* L

Other synonyms for this species are *Chamaesyce hirta* L., *Euphorbia pilulifera* L., *Euphorbia pilulifera* var. hirta L. Thell., *Euphorbia modiflora Steud*., *Euphorbia obliterata Jacq*., *Euphorbia verticillata* Vell., *Euphorbia capitata* Lam, *Euphorbia globulifera* (Al-Snafi, 2017; Or-Rashid et al., 2013).

The common names for *E. hirta* are as follows: asthma herb garden spurge, asthma weed, snakeweed, pill-bearing spurge, milkweed, hairy spurge, blotched leaf spurge, red euphorbia (English); ara tanah (Malay)(Al-Snafi, 2017).

E. hirta is an annual, broad-leaved, erect, ascending, or decumbent herb growing up to 50 cm, with hairy stems and milky latex. The leaves are opposite, simple (not lobed or split), elliptic, oblong, or oblong-lanceolate, with a toothed border and a darker top surface. The flowers are small, abundant, and crowded together in cymes, dense clusters in higher axils, about 1 cm in diameter; green or yellow in color. The fruits are yellow, three-celled, hairy, keeled capsules with three pinkish brown four-sided angular wrinkled seeds, measuring 1-2 mm in diameter. The roots are fleshy or tuberous, thick or fine (Al-Snafi, 2017; Patil et al., 2009). Figure 2 .1 and 2.2 show the plant sample and herbarium drawing of *E. hirta*.

Figure 2.1 *Euphorbia hirta* collected plant.

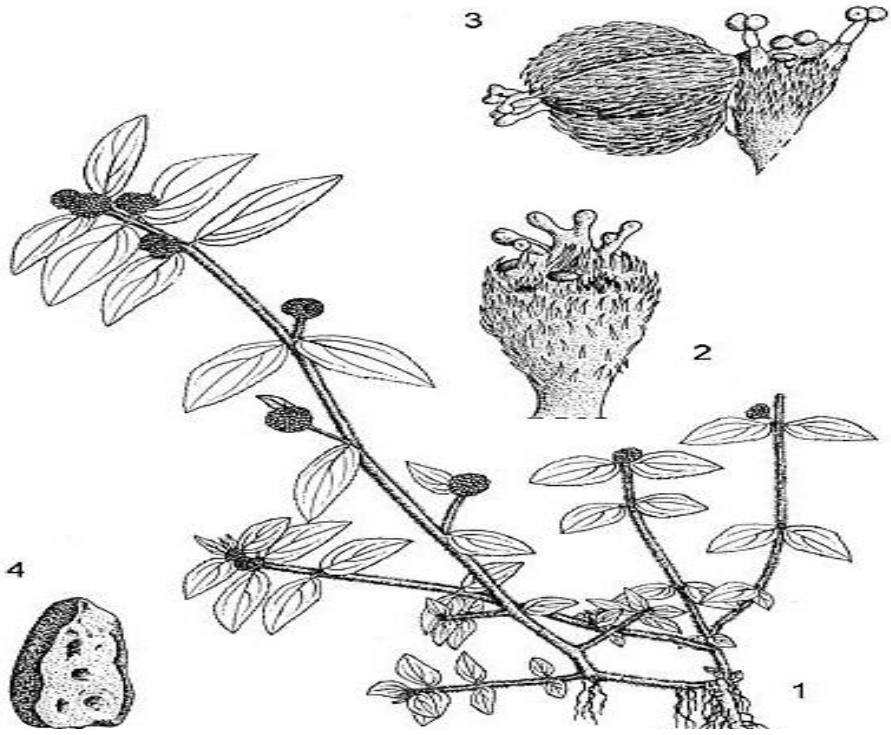


Figure 2.2 *Euphorbia hirta* plant parts;1: plant habit; 2: young cyathium; 3: mature cyathium; 4: Seed.

2.2.2(b) Traditional Uses of *Euphorbia hirta*

E. hirta was used to cure diarrhea, vomiting, intestinal parasites, acute enteritis, amoebic dysentery, peptic ulcers, and heartburn, among other things. (Kwan et al., 2013). It is also regarded as an excellent remedy for treating respiratory diseases such as asthma, emphysema, laryngeal spasms, bronchial infections, hay fever, dengue fever, coughs, and colds. (Emelike et al., 2015; Rao et al., 2010; Schmelzer et al., 2008). In addition, the plant is commonly used to treat urogenital illnesses such as kidney stones, menstruation issues, sterility, and venereal diseases. The plant's leaves are also used to treat skin and mucous membrane disorders such as aphthae, fungal infections, scabies, tinea, thrush, measles, and warts, as well as wounds, ulcers, and conjunctivitis (Abubakar, 2009; Jude-anthony et al., 2006; Rao et al., 2010). The plant is known for its use as an analgesic in the treatment of severe headaches, colic,

toothaches, rheumatism, and pregnancy symptoms. (Sood et al., 2005). It was also used to treat scorpion stings and snake bites as an antidote and pain reliever. It was used to cure worm infections in children, as well as diarrhea, gonorrhea, jaundice, acne, digestive issues, and tumors in India. The plant's roots were used in sprains and inflammation, miscarriage, epilepsy, maggots in wounds and irregular growth of teeth. Its use in the treatment of jaundice, oedema, hypertension, anemia and malaria (Igoli et al., 2005; Schmelzer et al., 2008).

2.2.2(c) Phytochemical Constituents

Phytochemical screening of *E. hirta* plant aerial part showed the presence of alkaloids, anthroquinones, reducing sugars, saponin, coumarin, tannins, terpenoids, steroids, proteins, glycoside, cardiac glycosides, flavonoids, phenolic compounds fats, oils, gums and mucilages. Table 2.3 shows the compounds that were reported to have been isolated from *E. hirta* with their classes. Figure 2.3 shows the structure of the selected compound that were reported to have been isolated from *E. hirta.*

Table 2.3 Compounds isolated from *Euphorbia hirta* plant.

Classes	**Isolated compounds**	**References**
Flavonoids	Quercetin Quercitrin Quercitol leucocyanidin leucocyanidol myricitrin kaempferol afzelin pinocembrin	(Al-Snafi, 2017)
Flavonoid glycoside	xanthorhamnin rutin isosinemsin cyanidin 3,5-diglucoside pelargonium 3,5-diglucoside quercetin-rhamoside luteolin-7-O-glucoside dimethoxyquercitrin 5,7,3′,4′ –trihyroxy-6-(3,3 –dimethyl allyl)-8-9-iso-butenyl)-flavonol-3-O-β-d-glucosidase quercetin-3-O-α-rhamnoside	(Al-Snafi, 2017)
Glycoside	n-butyl-1-O--L-rhamno- pyranoside	(Mallavadhani & Narasimhan, 2009)
Triterpenes	α-amyrin β-amyrin Friedelin Taraxerol Taraxerone Lupeol Cycloartenol Oxidotaraxerol Squalene α-amyrin acetate β-amyrin acetate α-amyrin fatty acid ester β-amyrin fatty acid ester cycloartenyl fatty acid ester lupeol fatty acid ester 11α, 12α-oxidotaraxerol 24-methylene-cycloartenol	(Hazimi A, 2008; Yan et al., 2011)

	taraxerol acetate j-taraxastane-3,20-diol friedelan-3b-ol 28-hydroxyfriedelin friedelane-3b,29-diol 3b-hydroxy-cycloart- 25-ene-24-one 23(E)-25-methoxycycloart-23-en-3b-ol cyclolanostan-3b-ol	
Diterpene	cycloart-23-ene-3b,25-diol cycloart-23-ene-3b,25,28-triol 12-deoxyphorbol-13-dodecanoate-20-acetate 12-deoxyp horbol-13-phenylacetate-20-acetate, ingenol triacetate tinyatoxin resiniferonol phytol	(Vasas & Hohmann, 2014; Yan et al., 2011)
Ent-kaurane diterpenoid	2-, 16-alpha,19-trihydroxy-ent-kaurane 2-,16- alpha-dihydroxy-ent-kaurane 16-alpha,19-dihydroxy- ent-kaurane	(Yan et al., 2011)
Sterols	β-sitosterol campesterol cholesterol stigmasterol β-sitosterol-D-glucoside	(Hazimi A, 2008)
Tannins	euphorbins A, B, C, E, terchebin geraniin (-)-epicatechin gallate (-)-epigallocatechin gallate 2,4,6-tri-O-galloyl-β-D-glucose 1,2,3,4,6- penta-O-galloyl-β-D-glucose neochlorogenic acid 3,4-di-O- galloylquinic acid benzyl gallate.	(Anand et al., 2016)
Long chain hydrocarbons	Hydrocarbon hentriacontane Tetradecane Myricyl alcohol Hexadecanal	(Al-Snafi, 2017)

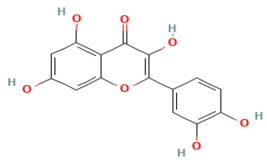
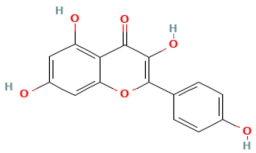
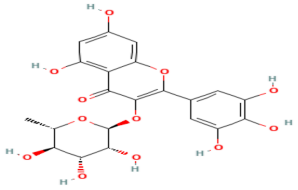
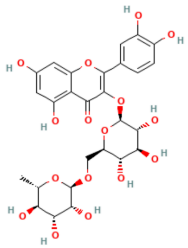
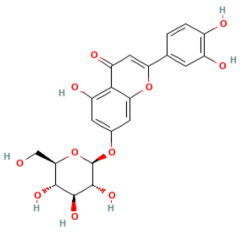
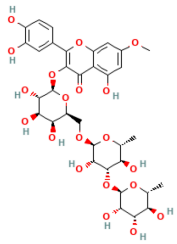
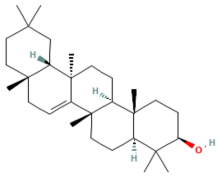
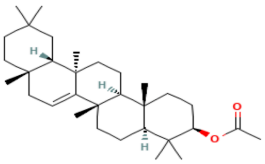
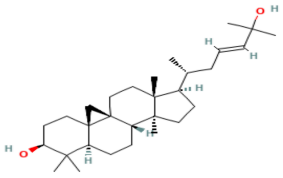
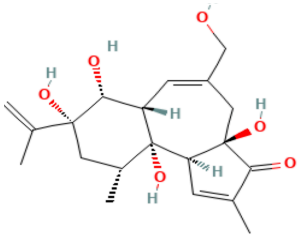
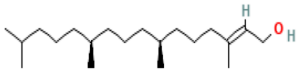
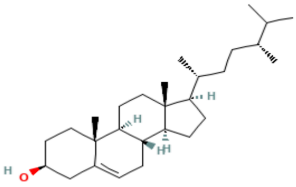
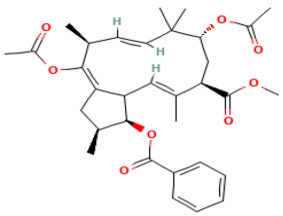
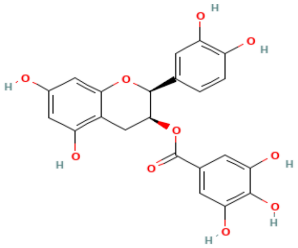
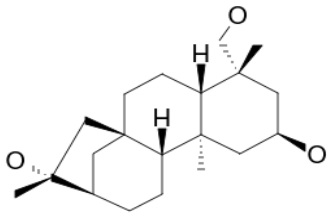
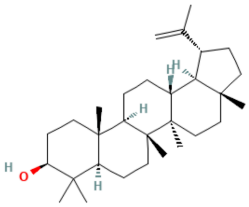
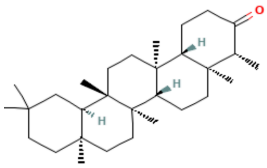
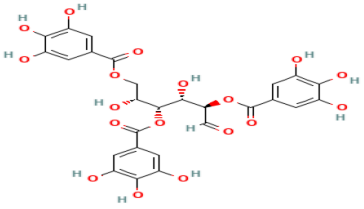
 Quercetin	 Kaempferol	 Myricitrin
 Rutin	 Luteolin-7-O-glucoside	 Xanthorhamnin
 Taraxerol	 Taraxerol acetate	 Cycloart-23-ene-3b,25-diol
 Resiniferonol	 Phytol	 Campesterol
 Euphorbin E	 (-)-epicatechin gallate	 2-β 16-α,19-trihydroxy-ent-kaurane
 Lupeol	 Friedelin	 2,4,6-tri-O-galloyl-β-D-glucose

Figure 2.3 The structure of the selected compounds isolated from *Euphorbia hirta*.

2.2.2(d) Pharmacological Activities

The plant of *E. hirta* has a long and illustrious history as a therapeutic herb throughout the world. A study reported early and late phase allergic responses were significantly reduced by *E. hirta* (Al-Snafi, 2017). Other research also reported the anti-anaphylactic property of *E. hirta* (Johnson et al., 1999; Singh et al., 2006; Youssouf et al., 2008). In an animal model study, the leaf extracts of *E. hirta* were reported to have a considerable influence on diuresis. It increased the urine output and electrolytes in rats (Johnson et al., 1999). Other studies that reported the pharmacological activities of this plant include anxiolytic, sedative, analgesic, antipyretic and anti-inflammatory (Lanhers et al., 1991), antifungal (Mohamed et al.,1996), antioxidant (Nilesh Sharma et al., 2007), antimicrobial (Abubakar, 2009; Jude-anthony et al., 2006), anthelmintic (Adedapo et al., 2004), antidipsogenic (Gossell-Williams et al., 1997), larvicidal (Al-Snafi, 2017), anticancer or antimutagenic (Sharma et al., 2014), antiasthmatic (Ekpo & Pretorius, 2008), galactogenic (Al-Snafi, 2017), antifeedant, antiplatelet aggregation and anti-inflammatory (Hiermann & Bucar, 1994), antiplasmodial (Tona et al., 2004), aflatoxin inhibition (Al-Snafi, 2017), antidiarrhoeal (Galvez et al., 1993), antiamoebic and spasmolytic (Tona, et al., 1998), repellent, antifeedant (Wei, et al., 2004), antimalerial (Liu et al., 2007), molluscicidal (Al-Snafi, 2017; Singh et al., 2005), and antidysenteric (Ridet & Chartol, 1964).

2.2.2(e) Wound Healing Studies of *Euphorbia. hirta*

Upadhyay et al. (2014) reported the *in vitro* antimicrobial, antioxidant and fibroblast proliferating activities of the methanolic extract of the leaves of the *E. hirta.*. It was reported that the extract showed significant proliferation activity (112% at 12

μg/mL) on fibroblast cells and anti-microbial activity against *Escherichia coli* and *Klebsiella pneumoniae*. The study also showed the alteration in the expression of proteins such as bFGF, COL3A1 and Smad which are important during granulation tissue in the wound repair process. (Upadhyay et al., 2014). The latter finding rationalized the traditional use of the *E. hirta* in wound healing

Another study by Bigoniya et al. (2013) reported the wound healing activity of flavonoid rich extract of *E. hirta* on rats. The wound healing activity was evaluated by the percentageof wound contraction, tensile strength, epithelisation period, and estimation of granulation tissue weight, hydroxyproline, superoxide dismutase (SOD), catalase and total protein content. The study found that the extract increased the tensile strength, wound contraction percentageand hydroxyproline content which provide strong evidence for would heal property of *E. hirta* flavonoid (Bigoniya et al., 2013).

A study by Rathnakumar et al. (2013) reported the wound healing activity of the ethanolic extract of the *E. hirta* leaves on rats using excision wound where .the extract showed significant ($p<0.001$) wound contraction. Furthermore, Jaiprakash et al. (2006) found that the ethanolic extract of *E. hirta* leaves showed significant burn wound healing activity on rat which justified the folklore used of this plant in burn management.

In addition, a study by Tuhin et al. (2018) reported the wound healing effect of *E. hirta* in alloxan-induced diabetic rats induced. . The histopathology of the wound tissues from excision wound that has been treated with *E. hirta* showed significant wound healing groups as compared to the diabetic control.

2.3 Wound Healing Models in Natural Product Research

Various models have been developed in the field of wound care research either *in silico, in vitro, in vivo*, and clinical (human) models.These models are important since they allowed for many different aspects of the healing process to be dissected and defined. The most frequently used models are described below.

2.3.1 *In vitro* Wound Healing Models

In vitro model for wound healing is a simple and efficient model that can be used regularly to provide evidence in support of wound care products (Justus, et al., 2014; Kramer et al., 2012). It can be beneficial as they are generally low-cost, rapid, and could be used to monitor a large number of samples at once. However, the disadvantage of the *in-vitro* assay is that the method is incapable of reproducing all components involved in complex wound healing processes (Au - Vang Mouritzen & Au - Jenssen, 2018). A common *in vitro* assay for wound healing is the scratch assay which uses cell lines. This model is used to investigate the impact of cell matrix, and cell-cell interactions on cell migration, which mimics cell migration during *in vivo* wound healing research.

Other *in vitro* assays that can be used to evaluate wound healing activity in natural product research are (Justus, et al., 2014; Kramer et al., 2012):

Cell proliferation assay; a test to determine if the number of cells in a test culture is growing when in the presence (or absence) of a test substance (e.g. chemical, material, etc.). Cell cytotoxicity assay; a test to determine if cells are killed by the presence of a

test substance. Cell viability assay; a test to determine if cells are able to maintain viability (i.e. remain alive) when exposed to a test substance.

Human dermal fibroblasts cells (HDFs) are used regularly in *in vitro* wound healing study as they are the most common cell type in connective tissues (Thakur, et al., 2011). Fibroblasts cells are able to produce a collagen scaffold during wound healing which are essential in wound healing process. During wound healing, the formation of collagen bundles is critical for the development of new blood vessels in the wounded areas. Fibroblasts also migrate to the wound area during the late inflammatory phase to proliferate and regulate various critical processes. Furthermore, fibroblasts also produce extracellular matrices and can differentiate into myofibroblasts, which help in wound healing (Xue & Jackson, 2015).

Cells contain a significant amount of antioxidants to prevent or repair the damage caused by the reactive oxygen species (ROS), as well as to regulate redox-sensitive signalling pathways. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) are three of the main antioxidant enzymes found in human cells that are regarded to be essential for survival in all oxygen-metabolizing cells. As seen in Figure 2.4, the SODs turn the superoxide radical into molecular oxygen and hydrogen peroxide (H_2O_2), meanwhile catalase and other peroxidases convert the hydrogen peroxide to oxygen and water. The GPx is the primary enzyme that breaks down the hydrogen peroxide into water. The interaction between the trio of free radicals, antioxidants, and the diseases is crucial, in order to retain health, delay aging and prevent age-related diseases. In fact, it has been proposed that lowering exposure to free radicals and increasing consumption of foods high in antioxidants or antioxidant supplements can improve the body's capacity to lessen the risk of health issues linked to free radicals (Ighodaro & Akinloye, 2018).

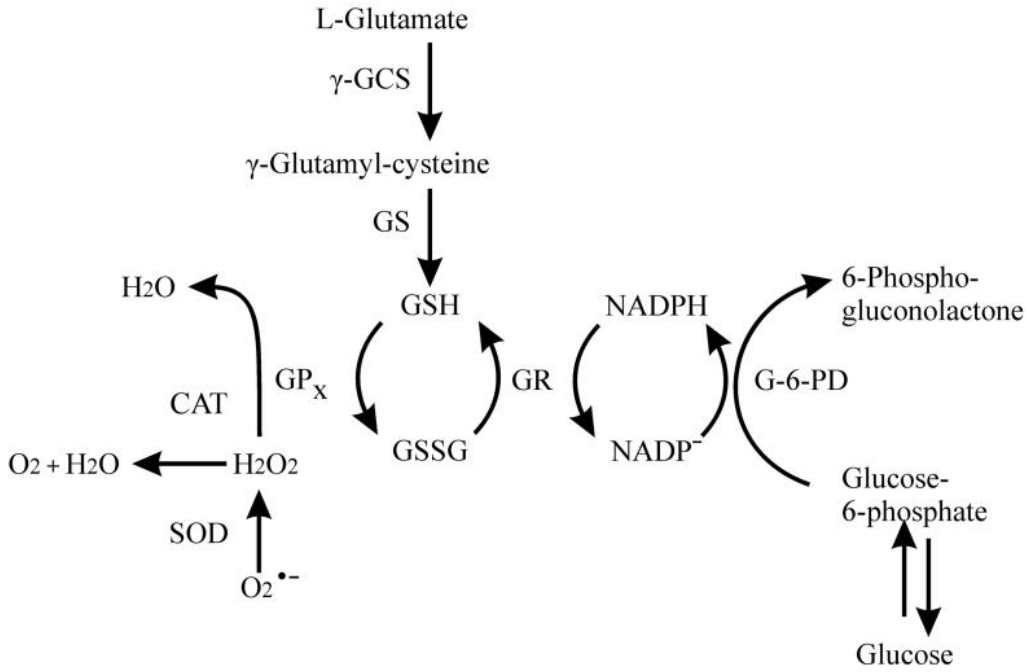


Figure 2.4 Antioxidant enzyme schematic.

The major types of primary intracellular antioxidant enzymes in mammalian cells SOD: Superoxide Dismutase; CAT: catalase; GPx: glutathione peroxidase. The SODs convert $O_2\bullet-$ into H_2O_2, whereas the CAT and GPx convert H_2O_2 into water. GPx requires several secondary enzymes to function in high efficacies, including, glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD), and cofactors, including glutathione (GSH), NADPH and glucose 6-phosphate. If GR is inhibited, cells cannot remove H_2O_2 through the glutathione peroxidase system, and the levels of glutathione disulfide (GSSG) increase. If glutathione synthesis is inhibited, either by inhibiting glutathione synthetase (GS) or by γ-glutamyl cysteine synthetase (γ-GCS), glutathione will be depleted and GPx will not be able to remove H_2O_2. If catalase is inhibited, cells also cannot remove H_2O_2. Finally, if glucose uptake is inhibited creating a chemically induced state of glucose deprivation, hydroperoxide detoxification will also be inhibited radicals (Ighodaro & Akinloye, 2018).