ANTIOXIDANT, ANTI-ANGIOGENIC AND ANTI-INFLAMMATORY ACTIVITIES OF STANDARDIZED *GYNURA SEGETUM* LEAF EXTRACTS

by

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABTS	(2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid))
AChE	Anti-cholinesterase
anti-CD31	Anti-cluster of differentiation 31
AP-1	Activating protein-1
bFGF	Basic Fibroblast Growth Factor
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BrdU	Bromodeoxyuridine
CAM	Chick embryo chorioallantoic membrane
CCD-18Co	Human colon fibroblast cell line
COX	Cyclooxygenase
CUPRAC	Cupric reducing antioxidant power
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
ELISA	Enzyme-linked immunosorbant assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FRAP	Ferric reducing antioxidant power
EC_{50}	Effective concentration 50
HET-CAM	Hen's egg chorioallantoic membrane test

HCT116	Human colon cancer
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factors
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IC_{50}	Inhibitory concentration 50
IFN	Interferon
iNOS	Inducible nitric oxide synthase
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
LTB_4	Leukotriene B_4
M	Molar
MCP1	Monocyte chemotactic protein-1
MMPs	Matrix metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	Number of samples
NaCl	Sodium chloride
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NO	Nitric oxide
NOS	Nitric oxide synthase

NSAIDS	Non-steroidal anti-inflammatory drugs
OD	Optical density
ORAC	Oxygen radical absorption capacity
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PG	Propyl gallate
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGH2	Prostaglandin H2
pH	Power of the concentration of the Hydrogen ion
PlGF	Placental Growth Factor
PLA_2	phospholipase A_2
r^2	correlation coefficients
R_f	Retardation factor
ROS	Reactive oxygen species
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor alpha
TRAP	Total Radical-Trapping Antioxidant Parameter
TWEEN	Polysorbate
U/mL	Units per milliliter

UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptors
v/v	Volume to volume
°C	Degree Celsius

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AKTIVITI ANTIOKSIDAN, ANTI-ANGIOGENIK DAN ANTI-INFLAMASI EKSTRAK TERPIAWAI DAUN *GYNURA SEGETUM*

ABSTRAK

Gynura segetum (daun dewa dalam bahasa Malaysia) ialah herba, dipercayai berguna untuk rawatan kanser dan inflamasi. Dalam kajian ini, aktiviti anti-inflamasi atas ekstrak terpiawai daun *Gynura segetum* dan juga kemungkian hubungan dengan aktiviti antioksidan, anti-angiogenik dan sitotoksik telah disiasat. Fraksi etil asetat menunjukkan aktiviti antioksidan yang terkuat berdasarkan asai memerangkap DPPH dan asai kapasiti pelunturan beta karotena dan berkaitan terus dengan jumlah kandungan fenolik dan flavonoid. Ekstrak metanol adalah yang paling aktif dalam menghalang pertumbuhan saluran darah dalam asai anak ayam embrio membran korioalantoik (CAM) dan asai aorta tikus cincin. Ini menunjukkan ia mempunyai aktiviti anti-angiogenik. Ekstrak metanol tidak sitotoksik dan penyekatan aktiviti faktor pertumbuhan endotel vaskular (VEGF) adalah salah satu mekanisme tindakan untuk anti-angiogenik. Ekstrak metanol mempunyai aktiviti anti-inflamasi kerana ia menghalang kerengsaan oleh SDS-teraruh dalam ujian telur ayam membran korioalantoik (HET-CAM) dan pembentukan granuloma dalam asai pelet kapas-teraruh granuloma pada tikus. Aktiviti anti-inflamasi ekstrak metanol ditunjukkan melalui perencatan pada tahap pro-inflammasi sitokin tumor nekrosis faktor-α (TNF-α), interleukin-1 (IL-1) dan enzim siklooksigenase-2 (COX-2). Oleh itu, penemuan daripada kajian ini mencadangkan bahawa daun *Gynura segetum* adalah sumber antioksidan semula jadi dan mempunyai aktiviti anti-angiogenik yang mungkin menjelaskan aktiviti anti-inflamasi kronik dan potensi dalam anti-kanser.

ANTIOXIDANT, ANTI-ANGIOGENIC AND ANTI-INFLAMMATORY ACTIVITIES OF STANDARDIZED *GYNURA SEGETUM* LEAF EXTRACTS

ABSTRACT

Gynura segetum (daun dewa in Malay) is a herb, reputed of value among others for treatment of inflammation and cancer. In the present study, the anti-inflammatory activity of the standardized leaf extracts of *Gynura segetum* were investigated as well as its possible relation to antioxidant, anti-angiogenic and cytotoxic activities. The ethyl acetate fraction showed the strongest antioxidant activity based on DPPH scavenging activity and beta carotene bleaching capacity assays and directly related to its total phenolic and flavonoid contents. The methanol extract was the most active in inhibiting the blood vessels growth in chick embryo chorioallantoic membrane (CAM) assay and rat aortic ring assay. It shows it has anti-angiogenic activity. It is non-cytotoxic and suppression of vascular endothelial growth factor (VEGF) activity is one of the anti-angiogenic mechanisms of action. The methanol extract possesses anti-inflammatory activity as it inhibited SDS-induced irritation in hen's egg chorioallantoic membrane (HET-CAM) test and cotton pellet-induced granuloma formation in rat. Its anti-inflammatory activity is mediated through the inhibition of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) and cycloxygenase-2 (COX-2) enzyme activities. Taken together, the present study suggests that *Gynura segetum*'s leaf is a natural source of antioxidants and possesses anti-angiogenic activity which may explain its chronic anti-inflammatory activity and anti-cancer potential.

CHAPTER 1

INTRODUCTION

1.1 Background

Herbal medicines are the therapeutic experiences of generations of practicing physicians of traditional medicine over hundreds of years and they are known to be oldest health care products that have been used by mankind all over the world to treat various types of ailments (Torey *et al*., 2010).

The demand for medicine from plants has increased and the global awakening of herbal medicines is also reflected in Malaysia. Medicinal plants grown in Malaysia have also received attention as new resources for alternative medicines over the past years (Sone *et al*., 2011). Currently, attention is being focused on the investigation of the efficacy of traditionally used plants as they are affordable and have fewer adverse effects (Masresha *et al*, 2012).

Gynura segetum, from family Compositae is a cultivated species and can be found growing in the tropical regions in Indonesia and Malaysia. *G. segetum* has drawn a lot of attention due to its uses in traditional medicine. The plant is known for its traditional use for the treatment of cancer, inflammation, diabetes, hypertension and skin afflictions. A variety of chemical compounds have been identified from this genus such as flavonoids, pyrolizidine alkaloids, triterpenes, steroids and sesquiterpenoids (Zhu *et al*., 2013). In spite of numerous phytochemical studies, there has been no systematic approach to understanding the ethno pharmacological activity of this plant.

In previous study, crude leaf extracts of *G. segetum* showed potential anti-angiogenic effect in chick embryo chorioallantoic membrane (CAM) model (Seow, 2010). Anti-angiogenic strategies are emerging as an important tool for the treatment of cancer and inflammatory diseases. Angiogenesis and inflammation are codependent processes, which involves both augmentation of cellular infiltration and proliferation and overlapping roles of regulatory growth factors and cytokines (Jackson *et al*., 1997). Many compounds that inhibit angiogenesis have also shown to block inflammatory responses due to this interconnection.

Angiogenesis in inflammatory diseases contribute to tissue growth, disordered tissue perfusion, abnormal ossification (formation of bony substance) and enhanced responses to normal or pathological stimuli (Walsh and Pearson, 2001). Angiogenesis inhibitor may reduce inflammation and also help to restore appropriate tissue structure and function. Most of the research on anti-angiogenic agents has been directed towards inflammation treatment (Kim *et al*., 2004; Ahn *et al*., 2007; Jung *et al*., 2009; Krenn and Paper, 2009). Collectively, this suggested that many herbs and their phytochemicals may exert both anti-angiogenic effects and anti-inflammatory activity. Thus, there is an interest to discover local medicinal plant for anti-angiogenic and anti-inflammatory effects.

Hence, the present study was carried out to expand the study on the anti-angiogenic and anti-inflammatory activities of the various extracts and fractions of *G. segetum*'s leaf, and investigate the probable mechanism of action of the most active extract/fraction. Standardization of the *G. segetum*'s leaf extract by chemical profiling (High performance thin layer chromatography), quantification of two

marker compounds using a validated HPLC (high performance liquid chromatography) method and biological profiling (determination of total phenolic and flavonoids content, and antioxidant activity) were also carried out. The experiments of the present study were summarized in Figure 1.1.

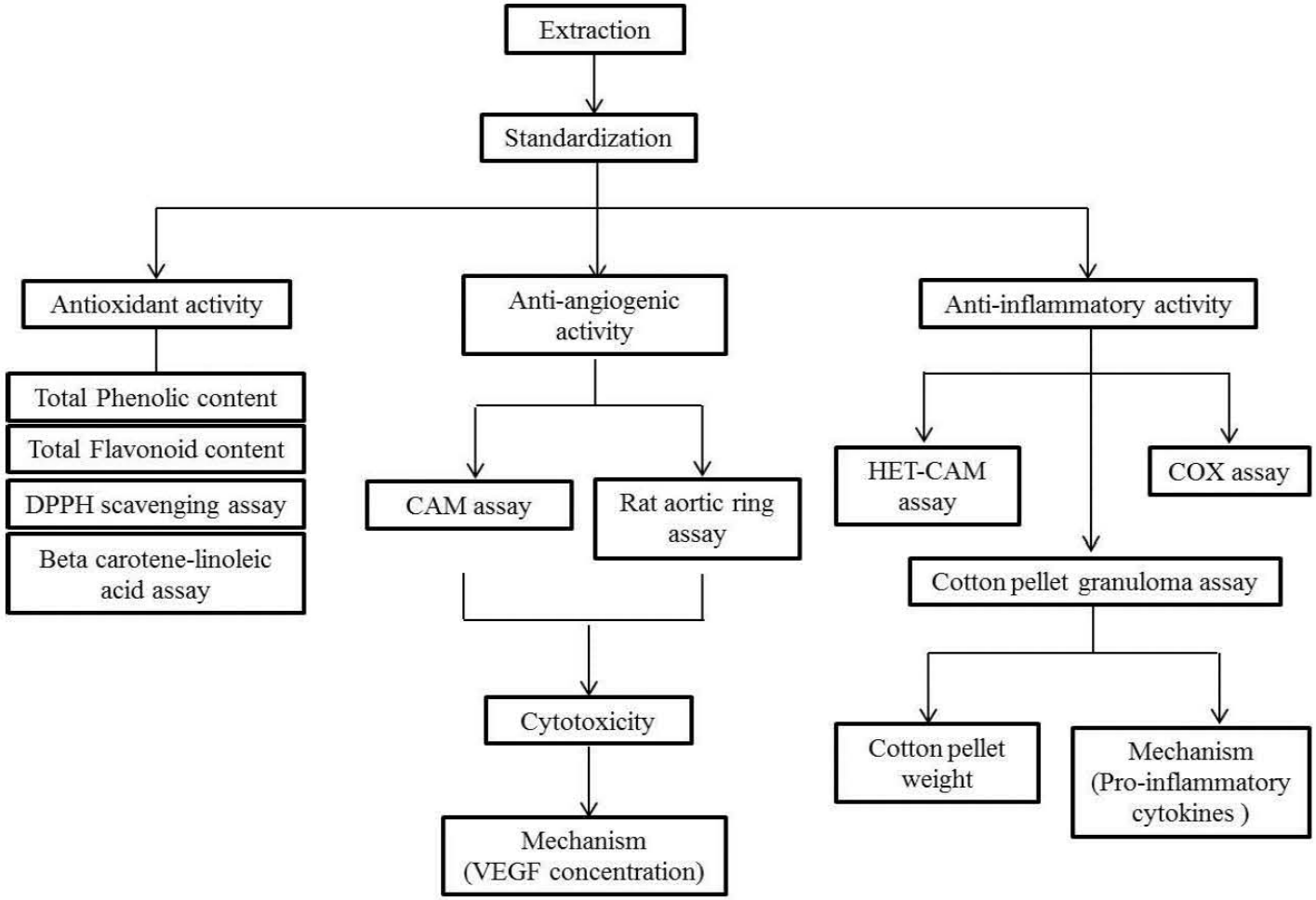

Figure 1.1: Flow chart of antioxidant, anti-angiogenic and anti-inflammatory activities of standardized *Gynura segetum* leaf extracts

1.2 Problem of Statements

Gynura segetum from family Compositae was claimed to possess various medicinal values including possible treatment of cancer, diabetes, inflammation and hypertension (Suharmiati and Maryani, 2003). Although this plant is traditionally used in treatment of cancer and inflammatory disorders, but to the best of our knowledge, its anti-cancer potential and anti-inflammatory activity has not yet been confirmed scientifically. The present studies focus on both *in vivo* and *in vitro* anti-angiogenic and anti-inflammatory activities in order to further investigate the potential of this plant. The standardization and antioxidant activities of the *G. segetum*'s leaf extract were also performed to evaluate its possible relation to the anti-angiogenic and anti-inflammatory activities.

1.3 Objectives

The objectives of the present study are:

1. To standardize the *G. segetum* leaf extracts by using phytochemical and chromatographic methods (HPTLC and HPLC).
2. To evaluate antioxidant potential of standardized leaf extracts of *G. segetum* by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity and beta-carotene-linoleate model assays and their correlation with total phenolic and flavonoid content.
3. To determine the anti-angiogenic activity of standardized leaf extracts of *G. segetum* by different experimental models (*in vivo* chicken chorioallantoic membrane assay and *ex vivo* rat aortic ring assay) and their possible mechanism of action.
4. To study the *in vivo* anti-inflammatory effects of standardized leaf extracts of *G. segetum* by different experimental models (hen's egg chorioallantoic membrane test and cotton pellet granuloma model in rats) and their possible mechanism of action.

CHAPTER 2

LITERATURE REVIEWS

2.1 Description of *Gynura segetum* (Lour.) Merr.

G. segetum from family Compositae is a cultivated species and can be found growing in the tropical regions in Indonesia and Malaysia. The leaves are shortly petiole, fleshy with serrated margin and both surfaces are pubescent (Figure 2.1). The upper surface is green and lower surface is purplish green. The stem branches profusely, purplish green in color and slightly hairy. The yellowish orange flowers have a strong and foetid odour. The plants produce numerous roots which are attached to a tuber. The plants collected from Jabatan Pertanian Relau, Penang were identified by the botanist (Mr. V. Shanmugan) from School of Biological Sciences, Universiti Sains Malaysia and a voucher specimen has been deposited at the herbarium of School of Biological Sciences, University Sains Malaysia with reference number 11013.

Figure 2.1: *Gynura segetum* (Lour.) Merr

2.2 Previous studies on *Gynura segetum*

A literature survey indicated that lack of research on *G. segetum* plant. To date, only few investigations on phytochemical studies of *G. segetum* have been reported. The structures of some isolated compounds of *G. segetum* are shown in Figure 2.2.

Alkaloid compound senecionine was elucidated and reported by Liang *et al*. (1984). Succinic acid, D-mannitol, thymine, adenine and pyrrolizidine alkaloids were isolated and identified by Liu *et al*. (1988) from aerial portion of *G. segetum* plant. Six alkaloids were isolated by Yuan *et al*. (1990) and four of them were identified as follows: senecionine, seneciphylline and seneciphyllinine. In Yang *et al*. (2009), a rapid reversed-phase high-performance liquid chromatographic (RP-HPLC) method was established for simultaneous determination of senecionice, seneciplylline and senecionine N-oxide in *G. segetum*.

Zhu *et al*. (2013) reported the isolation of nine new compounds from *G. segetum* for the first time. Their structures were identified to be stigmasterol, isoarborinol, arborinol, zhebeiresinol, lumichrome, (2*S*,3*S*,4*R*,8*E*)-2-[(2*R*)-2-hydroxypalmitoylamino]-8-octadecene-1.3.4-triol, syringic acid, vanillic acid, *trans*-p-hydroxycinnamic acid on the basis of mass and NMR spectra.

Previous study demonstrated that the leaf extracts of *G. segetum* inhibited the sprouting of blood vessels growth in chick embryo chorioallantoic membrane (CAM) model. The anti-angiogenic activity of *G. segetum* provided a pharmacological basis on its folkloric

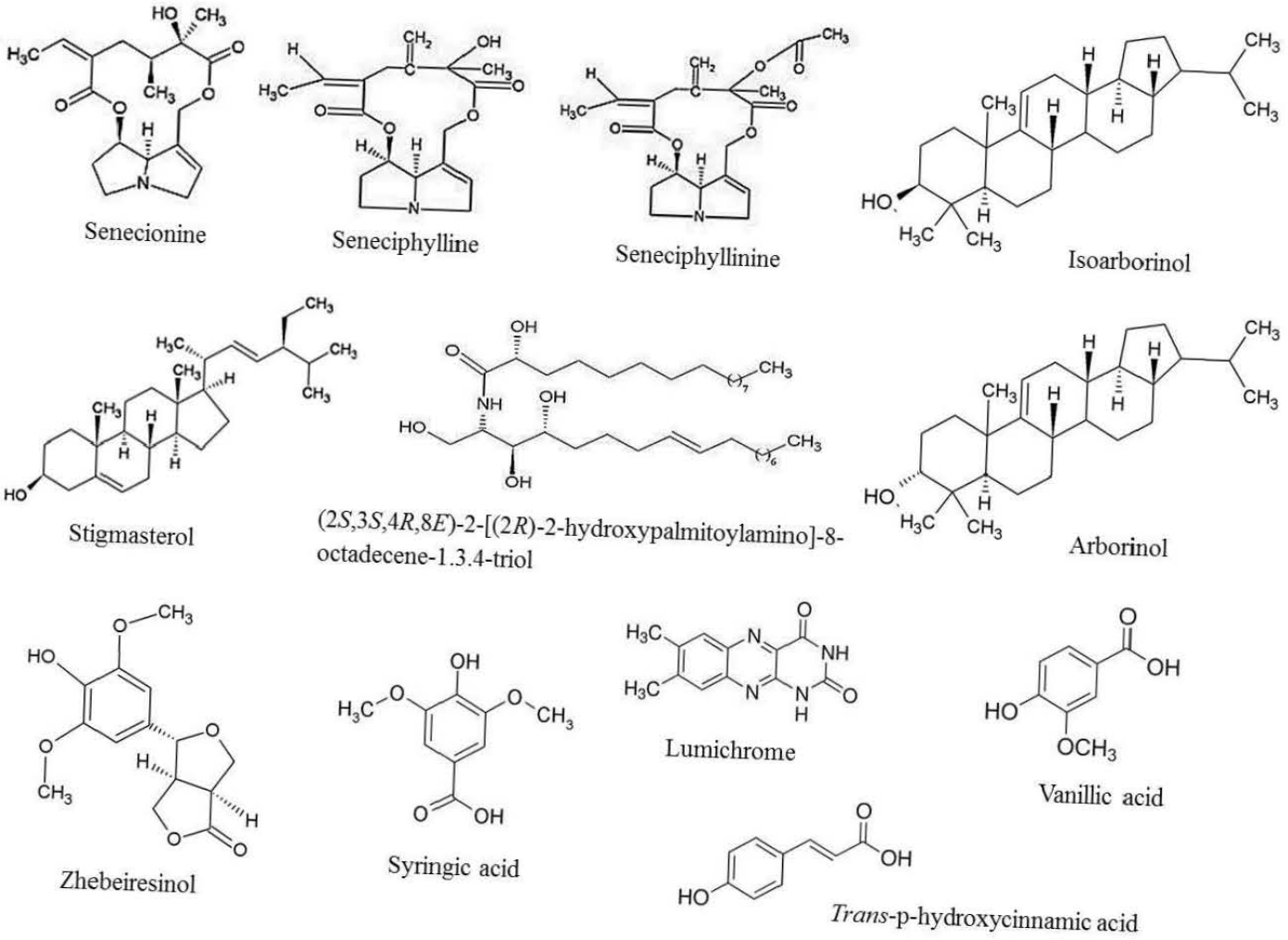

Figure 2.2: Chemical structures of some isolated compounds of *Gynura segetum*

use for the treatment of inflammatory diseases and cancer (Seow, 2010). Angiogenic factors have a central role in promoting cancer and a strategic position in the regulation of angiogenesis, eventually stimulating endothelial cells that are genetically stable in comparison with the genetically labile tumor cells (Schenone *et al*., 2007). New blood vessels can maintain the chronic inflammatory state by transporting inflammatory cells to the site of inflammation and supplying nutrients and oxygen to the inflamed tissue. The inflamed tissues are induced to release large quantities of angiogenic factors (VEGF) and cytokines (Walsh and Pearson, 2001). Therefore, anti-angiogenic therapy is a promising approach for the treatment of cancer and other angiogenesis-dependent diseases i.e. chronic inflammation.

Preliminary phytochemical screening revealed the presence of alkaloids, terpenes, flavonoids, tannins and saponins in leaves of *G. segetum* (Seow, 2010). On the other hand, the presence of phenolic compounds in the plants indicates that this plant may be anti-microbial agent and this agreed with the findings of Seow (2010). Moreno *et al*. (2006) suggested that the antimicrobial action of phenolic compounds was related to inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell or caused by membrane permeability changes. Increased membrane permeability is a major factor in the mechanism of antimicrobial action, where phenolic compounds disrupt the membranes and causing loses of membrane permeability and eventually the death of cell (Cetin-Karaca, 2011).

2.3 Standardization

Standardization of herbal medicines means confirmation of its identity and determination of its quality and purity (Pandey and Tripathi, 2014). Plants contain phytochemical that can be used for therapeutic purpose or as precursors for pharmaceutical synthesis. The world market demand for herbs and their products has increased tremendously in recent years. Therefore, methods for standardization of herbal raw materials and herbal formulations are developed.

Pharmacopoeias carry monographs for herbs and herbal products to maintain their quality. Several pharmacopoeias including United States Pharmacopoeia, Indian Pharmacopoeia, European Pharmacopoeia, and British Pharmacopoeia do cover monographs and quality control tests for a few medicinal plants. There many changes are being incorporated into the different pharmacopoeias, as reflected by the availability, development, and subsequent refinement of analytical techniques (Rajani and Kanaki, 2008). In the present study, the standardization and validation of analytical procedures were carried out according to the European Pharmacopoeia.

Standardization of herbal medicines involves the collection of information and application of strict quality control measurement from the growing of a medicinal plant to the finished therapeutic substance (Garg *et al*., 2012). The standardization of plant includes a complete description of the starting plant materials, control and monitoring of numerous factors namely growing conditions, harvesting time, harvested plant part, microscopy and macroscopic analysis, ash values and extractive values, moisture content determination, drying methods, storage, extraction process, chromatographic and analytical evaluation which are required for providing

consistency of the quality of an plant extract (Bele and Khale, 2011; Garg *et al*., 2012).

Most of the medicinal plants have definite specific chemical constituents to which their biological or pharmacological activity is attributed (Pandey and Tripathi, 2014). Pharmacological properties of a plant extract depend on phytochemical constituents present therein. Without a consistent quality of a phytochemical mixture, a consistent pharmacological effect is not achieved (Mosihuzzaman and Choudhary, 2008; Maiti *et al*., 2011). Phytochemical standardization comprises all the possible information with regard to the chemical constituents present in herbal medicines. The phytochemical evaluations include preliminary screening for the presence of different chemical groups (e.g. alkaloids, flavonoids, saponins, triterpenic acids, tannins and etc.), quantification of chemical of interest and establishment of fingerprint profiles based upon single or multiple markers (Kulkarni *et al*., 2014).

Chromatographic fingerprinting plays an important role in the quality control of complex herbal medicines (Kamboj and Saluja, 2013). A fingerprint refers to the common peaks of chromatograms which can be used to characterize and determines the concentration of a set of characteristic chemical substances in an herb (Kulkarni *et al*., 2014). Using this technique, the identification of various chemical markers of the herbal medicines can be easily done and it also serves as a tool for authentication and quality control of herbal drugs (Kasar *et al*., 2013).

Chromatographic fingerprint of herbal medicine is a chromatographic pattern produced from extract of some specific chemical constituents which may be

pharmacologically active or have some chemical characteristics (Kulkarni *et al*., 2014). Standardized extracts are high-quality extracts containing consistent levels of specified marker/bioactive compounds (Bandaranayake, 2006). There are two types of standardized extract, which are active constituent extract and marker compound extract. Active constituent extract contains the constituents with known therapeutic effect which can be used to standardize a biological activity. However, marker compound is not necessarily active compounds, as chemically defined constituents, which are used for control purpose (Kamboj and Saluja, 2013).

Sensitivity is the major concern for the detection of various chemical markers in extract. Thus, chromatographic techniques in combination with different detections are the preferred techniques. High performance thin layer chromatography (HPTLC) and High performance liquid chromatography (HPLC) are suitable analytical method for the separation and quantitative determination of various chemical markers of herbal medicines.

2.3.1 High performance thin layer chromatography (HPTLC)

HPTLC has recently emerged as an useful tool for fingerprinting and quantification of marker compounds in herbal medicines (Bala and Saini, 2013). In its traditional form, thin layer chromatography has use in identification of botanical raw materials (Kasar *et al*., 2013). The modern HPTLC offers better resolution which allows a quantitative analysis at micro and even in nano gram levels (Kamboj and Saluja, 2013). A wide spectrum of constituents can be detected at the same time in a single run in an experiment by HPTLC and the flexibility of detection are particularly useful for fingerprinting analysis.

The modern HPTLC can generate a chromatographic fingerprint in the form of an unique sequence of peaks corresponding to the analyzed sample in its fullness (Nicoletti, 2011). The resulted chromatogram can be evaluated based on number, sequence, color and relative position of zones (R_f) with respect to zone obtained on the same plate with chemical reference materials (Kasar *et al*., 2013). The results are not only reported as peak data, but can also conveniently be presented as colorful images, their absorption spectra, λ max and shoulder inflections of all the resolved bands (Kamboj and Saluja, 2013).

Collectively, fingerprinting of HPTLC is proved to be a linear and precise method for herbal identification and can be further used in authentication and characterization of the medicinally important plant (Sushma *et al*., 2013). However, HPTLC method has few limitations, such as the limited developing distance and it is often not possible to obtained baseline separation of all components of such complex samples as medicinal plant materials (Kasar *et al*., 2013). Also, the sensibility of

HPTLC is still not comparable with HPLC. In HPTLC, two closely related compounds may not be appropriately resolved and hence migrate as a single peak. This situation would result into misleading results in quantitative analysis, especially if one of the non-resolved compounds is an unknown impurity or a degradation product (Hewala *et al*., 2012). Therefore, most fingerprinting analysis is typically based on HPLC.

2.3.2 High performance liquid chromatography (HPLC)

HPLC is one of the most important tools of analytical chemistry and widely used for fingerprinting study of herbal plants. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase through the column, and a detector that shows the retention times of the molecules (Malviya *et al*., 2010). The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behavior of different components, elution at different time takes place (Gupta *et al*., 2012).

Analytical method development and validation are key elements of any pharmaceutical development program (Singh, 2013). HPLC analysis method is developed to ensure the identity, purity and quantification of the chemical constituents in herbal plant. All analytical methods that are intended to be used for analyzing any chemical constituents in plant material will need to be validated (Gupta *et al*., 2012). The validation practice demonstrated that an analytic method measures the correct constituent in correct quantity, and in the proper range for the intended samples (Julia *et al*., 2011; Khan *et al*., 2012).

The validation of analytical method is performed according to the ICH (International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) guidelines (ICH, 2002). The various validation parameters include linearity, accuracy, limits of detection and quantification (LOD and LOQ), precision, robustness and selectivity or specificity (Singh, 2013).

Reproducible quality of HPLC results can only be obtained if proper attention has been paid to the method development, validation and system's suitability to carry out the analysis (Singh, 2013). The validated methods provide emerging knowledge supporting the standardization of herbal plant and the method could be utilized for quality check of various pharmaceutical and nutraceutical preparations developed from the herbal plants (Masresha *et al.*, 2012). The herbal plants will be earlier tested in animal experiments and further tested in a clinical study in order to prove its safety and efficacy.

2.4 Antioxidant

Antioxidants are defined as molecules which can be safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Nidhi and Gauray, 2012). Antioxidants can also be molecules which prevent or inhibit free radical formation, involved in the defense mechanism of the organism against the pathologies associated to the attack of free radicals (Pisoschi and Negulescu, 2011).

Antioxidants can be derived from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), but can also be synthetic compounds, like butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), etc (Pisoschi and Negulescu, 2011). BHA and BHT are widely used in food industry as antioxidants in order to reduce the damage caused by free radicals. However, the toxicological research demonstrated that these synthetic antioxidants possess toxic, pathogenic and carcinogenic effects (Ganie *et al.*, 2011; Jiang *et al.*, 2013). Therefore, interest in finding natural antioxidants from plant materials has drawn more and more attention (Safaei-Ghomi *et al.*, 2009).

The use of traditional medicine is widespread and plants are potential source of natural antioxidants that might serve as leads for the development of novel drugs (Conforti *et al.*, 2008). Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, enzyme inhibitors and synergists (Lai and Palanisamy, 2012). Antioxidants such as the vitamins A, C and E, beta carotene, flavonoids, mineral selenium have potential to prevent oxidative damage and demonstrated their potent antioxidant activities both

in vitro and *in vivo* (Mokhtar *et al*., 2008; Pandey and Rizvi, 2009; Hamid *et al*., 2010).

2.4.1 The mechanism of actions of antioxidant

Free radicals are considered as unstable molecule, which react quickly with other compounds, trying to capture the needed electron to gain stability (Nidhi and Gauray, 2012). Free radicals of highly reactive oxygen species (superoxide, $O_2^{\bullet -}$; hydroxyl, $OH^{\bullet}$; peroxyl, $ROO^{\bullet}$; peroxinitrite, $^{\bullet}ONOO^-$ and nitric oxide, $NO^{\bullet}$) produced through oxidative process (Abdel-Hameed, 2009). Oxidative stress occurs when there is an imbalance between free radical reactions and the scavenging capacity of antioxidative defense mechanism of the organism (Seifu *et al*., 2012). A number of biochemical processes can initiate oxidation, which proceeds continuously in the presence of (a) suitable substrate(s), until a blocking defense mechanism occurs (Pisoschi and Negulescu, 2011).

Antioxidants are compounds or systems that delay autoxidation by inhibiting the formation of free radicals by several mechanisms: initiation, propagation, and termination (Hamid *et al*., 2010) as shown below. The oxidation may be initiated by the action of external agents such as heat, light or ionizing radiation or by chemical initiation involving metal ions or metalloproteinase (Pisoschi and Negulescu, 2011).

a) Initiation: free radicals are formed from molecules that readily give up electrons.

(1) $RH \rightarrow R^{\bullet} + H^{\bullet}$

(2) $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

(3) $2ROOH \rightarrow ROO^{\bullet} + RO^{\bullet} + H_2O$

b) Propagation: the free radicals are alternately expended and formed.

(1) $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

(2) $ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

(3) $RO^{\bullet} + RH \rightarrow ROH + R^{\bullet}$

c) Termination: involve the combination of radicals to form non-radical products.

(1) $R^{\bullet} + R^{\bullet} \rightarrow R - R$

(2) $R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$

(3) $ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$

2.4.2 Methods of antioxidant activity assessment

Antioxidants affect the process of lipid oxidation at different stages due to the differences in their mode of action (Ghasemzadeh *et al*., 2010). There is no single method that can provide unequivocal measurement of antioxidant activity and the best solution is to use various methods instead of a one-dimension approach (Carocho and Ferreira, 2013).

a) The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method

The ABTS assay is a colorimetric assay in which the ABTS radical decolorizes in the presence of antioxidants (carotenoids, phenolic compounds and others) (Moon and Shibamoto, 2009). The ABTS cation radical (ABTS•+) which absorbs at 743 nm (giving a bluish-green colour) is formed by the loss of an electron by the nitrogen atom of ABTS. In the presence of hydrogen donating antioxidant, the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization (Pisoschi and Negulescu, 2011).

b) The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method

DPPH (2,2-diphenyl-1-picrylhydrazyl) is characterized as stable free radical and able to accept an electron or hydrogen radical (Kchaou *et al*., 2013). This colorimetric assay uses the DPPH radical, which changes from purple to yellow in the presence of antioxidants (Moon and Shibamoto, 2009). When DPPH reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet color. Therefore, the absorbance diminution depends linearly on the antioxidant concentration (Pisoschi and Negulescu, 2011).

c) The ferric reducing antioxidant power (FRAP) method

The FRAP method is characterized by the reduction of iron, Fe^{3+} to Fe^{2+} depending on the available reducing species followed by the alteration of color from yellow to blue and analyzed through a spectrophotometer (Antolovich *et al*., 2002). Reducing power appears to be related to the extent of conjugation in phenols as well as the number of hydroxyl constituents (Schaich, 2006). Further, the assay reaction must be carried out at acidic pH in order to maintain iron solubility (Craft *et al*., 2012).

d) The oxygen radical absorption capacity (ORAC) assay

This method measures the antioxidant scavenging activity against the peroxyl radical ($ROO^{\bullet}$), induced by 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH), at 37ºC. Fluorescein is used as the fluorescent probe. The loss of fluorescence is an indicator of the extent of the decomposition, from its reaction with the peroxyl radical (Thaipong *et al*., 2006; Denev *et al*., 2010). Any antioxidant species present in the reaction mixture will undergo hydrogen atom transfer mechanism with the peroxyl radicals and delay the reduction of the fluorescent signal (Craft *et al*., 2012).

e) The total radical-trapping antioxidant parameter (TRAP) assay

The TRAP assay is used to determine the total antioxidant activity, based on measuring oxygen consumption during a controlled lipid oxidation reaction induced by thermal decomposition of AAPH (2,2'-Azobis(2amidinopropane)hydrochloride) (Antolovich *et al*., 2002). The basic reactions of the assay are similar to those of ORAC. The TRAP assay involves the initiation of lipid peroxidation by generating water-soluble peroxyl radicals and is sensitive to all known chain breaking antioxidants, but it is relatively complex and time-consuming to perform, requiring a high degree of expertise and experience (Prior *et al*., 2005).

f) The cupric reducing antioxidant power (CUPRAC) assay

The method relies on the reduction of copper, Cu (II) to Cu (I) by the antioxidants present in the sample. Then, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) forms a complex with Cu (I) and has a maximum absorbance at 490 nm (Huang *et al*., 2005). Copper has a lower redox potential than iron, so its reactions are more selective e.g. sugars and citric acid which commonly interferences with iron in FRAP, are not oxidized in CUPRAC. The low redox potential enhances redox cycling, so copper reduction may be an even more sensitive indicator of potential pro-oxidant activity of antioxidants (Prior *et al*., 2005).

g) Beta-carotene linoleic acid method/conjugated diene assay

The basis of the beta-carotene bleaching assay is the discoloration of beta-carotene in a reaction with a linoleic acid free radical. A radical is formed upon the removal of a hydrogen atom located between two double bonds in linoleic acid. Beta-carotene has

a maximum absorbance at 470 nm. Antioxidants present in the reaction solution can prevent the degradation of beta-carotene (Fu *et al*., 2013).

2.4.3 Herbal medicines and antioxidant activities

To date, numerous of plants in Malaysia have been studied for their antioxidant activity. *Garcinia mangostana*, *Mangifera indica*, *Nephelium lappaceum*, *Anacardium occidentale*, *Piper betle* and *Syzygium aquem* exhibit high antioxidant activity and proposed to be an alternative source of polyphenols (Mokhtar *et al*., 2008; Lai and Palanisamy, 2012). Compounds that have several or many phenolic hydroxyl substituents are often referred to as polyphenols, which have strong hydrogen atom donating activity to terminate the chain reactions of free radicals and inhibit oxidation (Maestri *et al*., 2006).

An antioxidant activity has been reported in a number of *Gynura* species. Caffeic acid and quercetin 3-O-rutinoside isolated from *Gynura formosana Kiamnra* were found to show good antioxidant activity against DPPH and hydroxyl radicals (Hou *et al.*, 2005). The antioxidant potency of *Gynura procumbens* leaves were investigated by Rosidah *et al.* (2008); Akowuah *et al.* (2009) and Shwter *et al.* (2014), suggest that phenolic compounds in these plants provide substantial antioxidant activity. The *Gynura medica* leaf extract showed a moderate radical scavenging activity, which agreed with the moderate level of flavonoid compounds existing in the plant; kaempferol-3-O-glucoside was the major compound of the plant (Liu *et al.*, 2010). Meanwhile, the ethyl acetate extract of *Gynura bicolor* with the highest total phenolic content among these species extracts generally showed the strongest ability in scavenging DPPH radicals and metal chelating activity (Teoh *et al.*, 2013).

2.5 Angiogenesis

Angiogenesis is a progressive, multistep physiological process by which new blood vessels are generated from pre-existing vasculature (Deryugina and Quigley, 2008). Angiogenesis plays a central role in various physiological processes within human body, such as, embryonic development, menstrual cycle, and wound repair. Unregulated angiogenesis is intensively involved in many pathological conditions including psoriasis, retinopathy and cancer and various ischemic and inflammatory diseases (Choe and Lee, 2007; Yoo and Kwon, 2013).

The process of angiogenesis can be simply described into three major steps including:

i) Initiation of the angiogenic response: Angiogenesis is initiated in response to hypoxia, by the release of hypoxia inducible factors (HIF), which facilitate the release of angiogenic stimulators, which in turn lead to endothelial cell activation. Activated endothelial cell secrete matrix metalloproteinases (MMPs) and plasminogen activators (PAs), which degrade the extracellular tissue to facilitate endothelial penetration. Both PAs and MMPs are secreted together with their inhibitors, ensuring a stringent control of local proteolytic activity (Bisht *et al*., 2010).

ii) Endothelial cell migration, proliferation and tube formation: Extracellular matrix degradation results in an increased concentration of various growth factors, which stimulate endothelial cell migration and proliferation. After the initial period of migration, rapid endothelial cell proliferation begins, thus increasing the rate of sprout elongation. These processes are also mediated by cell adhesion

molecules (Integrin $\alpha_v\beta_3$and $\alpha_v\beta_{5,}$ vascular endothelial cadherin, vascular cell adhesion molecule-1, P-selectin, and E-selectin) (Bisht *et al*., 2010).

iii) Maturation of the neovasculature: The final phase of the angiogenic process involves maturation of the neovasculature. After endothelial cell proliferation and maturation, and the formation of endothelial tube structures, surrounding vessel layers composed of mural cells (pericytes in small vessels and smooth muscle cells in large vessels), need to be recruited. Endothelial cells may accomplish this via the synthesis and secretion of platelet-derived growth factor (PDGF), a mitogen and chemo attractant for a variety of mesenchyme cells. Subsequent differentiation of the mural precursor cells into pericytes and smooth muscle cells is believed to be a cell-cell contact dependent process (Griffioen and Molema, 2000). Finally, when sufficient neovascularization has occurred, the angiogenic factors are down regulated or the local concentration of the inhibitors increases. As a result, the endothelial cells become quiescent (Bisht *et al*., 2010).

2.5.1 Angiogenic factors and inhibitors

Angiogenesis is a tightly controlled by a complex balance between the pro-angiogenic and anti-angiogenic factors (Tahergorabi and Khazaei, 2012). A number of endogenous angiogenic factors and inhibitors have been discovered and identified, that regulate the balance between the pro- and anti-angiogenic states (Carmeliet *et al*., 2000). Some of the most important factors and inhibitors are shown in Table 2.1.