CHAPTER II

LITERATURE REVIEWS

2.1 Botanical aspects of *Lagerstroemia speciosa* (L.) Pers.

The genus *Lagerstroemia* belongs to the family Lythraceae. *Lagerstroemia* species are distributed in tropical and subtropical parts of Madagascar, Asia, New Guinea and Australia (Everett *et al.*, 1972). There are at least eighteen valid species of *Lagerstroemia* in Thailand according to Smitinand (2001), as listed in **Table 2.1**.

Leaves of the tropical plant, *Lagerstroemia speciosa* , have been used as a folk medicine for treatment of diabetes (Lui *et al.*, 2001). *L. speciosa* was distributed in China, Indo-china, Burma, Siam, Peninsular Malaysia, Sumatra, Java, Celebes, Perak, Pahang southwards, Langkawi, and the Philippines (Matsuyama, 2001; Quisumbing, 1978). There ecology was in lowland secondary forest on sandy soil. In Flora of Thailand, there is no information about *Lagerstroemia* characteristics. The Tree Flora of Sabah and Sarawak (Yii, 1996) described the characteristic of *L. speciosa* as follows;

"Tree to 10 m tall, 30 cm diameter. Leaves elliptic-oblong, 5 – 19 x 4 – 8 cm; base cuneate or almost rounded, apex acute or obtuse; glabrous, grayish green above and brown beneath; lateral veins 8-15 pairs, distinctly looped and joined to the next pair; petioles 4-9 mm long. Inflorescences 15 – 40 cm long, 10 – 20 cm across, covered with caduceus, ashy or rusty hairs; pedicels c. 1.5 mm long. Flower buds subglobose or pyriform, 10 – 15 x 6 – 10 mm, shortly apiculate at the apex, 12 – 14-ridged, ridges of the same length; calyx ashly or rusty hairy, lobes recurved; petals 6, suborbicular, 15 – 30 x 10 – 20 mm, tapering towards the slender claw; stamen numerous, subequal; ovary globose, glabrous or slightly scaly, style filiform, stigma capitates. Fruits woody, globose, glabrous, c. 22 mm across; persistent calyx glabrous or slightly hairy, lobes recurved."

Table 2.1 The list of *Lagerstroemia* species in Thailand.

No.	Scientific name	Thai name
1	*Lagerstroemia balansae* Koehne.	Ta baek kriap (ตะแบกเกรียบ)
2	*L. calyculata* Kurz.	Ta baek daeng (ตะแบกแดง)
3	*L. collinsae* Craib.	Ta baek bai lek (ตะแบกใบเล็ก)
4	*L. cuspidate* Wall.	Ta baek (ตะแบก)
5	*L. duperreana* Pierre ex Gagnep.	Ta baek plueak bang (ตะแบกเปลือกบาง)
6	*L. floribunda* Jack.	Ta baek na (ตะแบกนา)
7	*L. indica* L.	Yi Kheng (ยี่เข่ง)
8	*L. loudinii* Teijsm. & Binn.	In thara chit (อินทรชิต)
9	*L. macrocarpa* Wall.	Inthanin bok (อินทนิลบก)
10	*L. ovalifolia* Teijsm. & Binn.	Ta baek dong (ตะแบกดง)
11	*L. siamica* Gagnep.	Ta baek na (ตะแบกนา)
12	*L. speciosa* (L.) Pers.	Inthanin nam (อินทนิลน้ำ), (syn. *L. flos-reginae* Retz.)
13	*L. spireana* Gagnep.	Pueai nam (เปื๋อยน้ำ)
14	*L. tomentosa* C.Presl	Salao Khao (เสลาขาว)
15	*L. undulata* Koehne var. undulate	Salao dam (เสลาดำ)
16	*L. undulata* Koehne var. subangulata Craib.	Samo rong (สมอร่อง)
17	*L. venusta* Wall.	Salao plueal bang (เสลาเปลือกบาง)
18	*L. villosa* Wall. ex Kurz	Salao plueak na (เสลาเปลือกหนา)

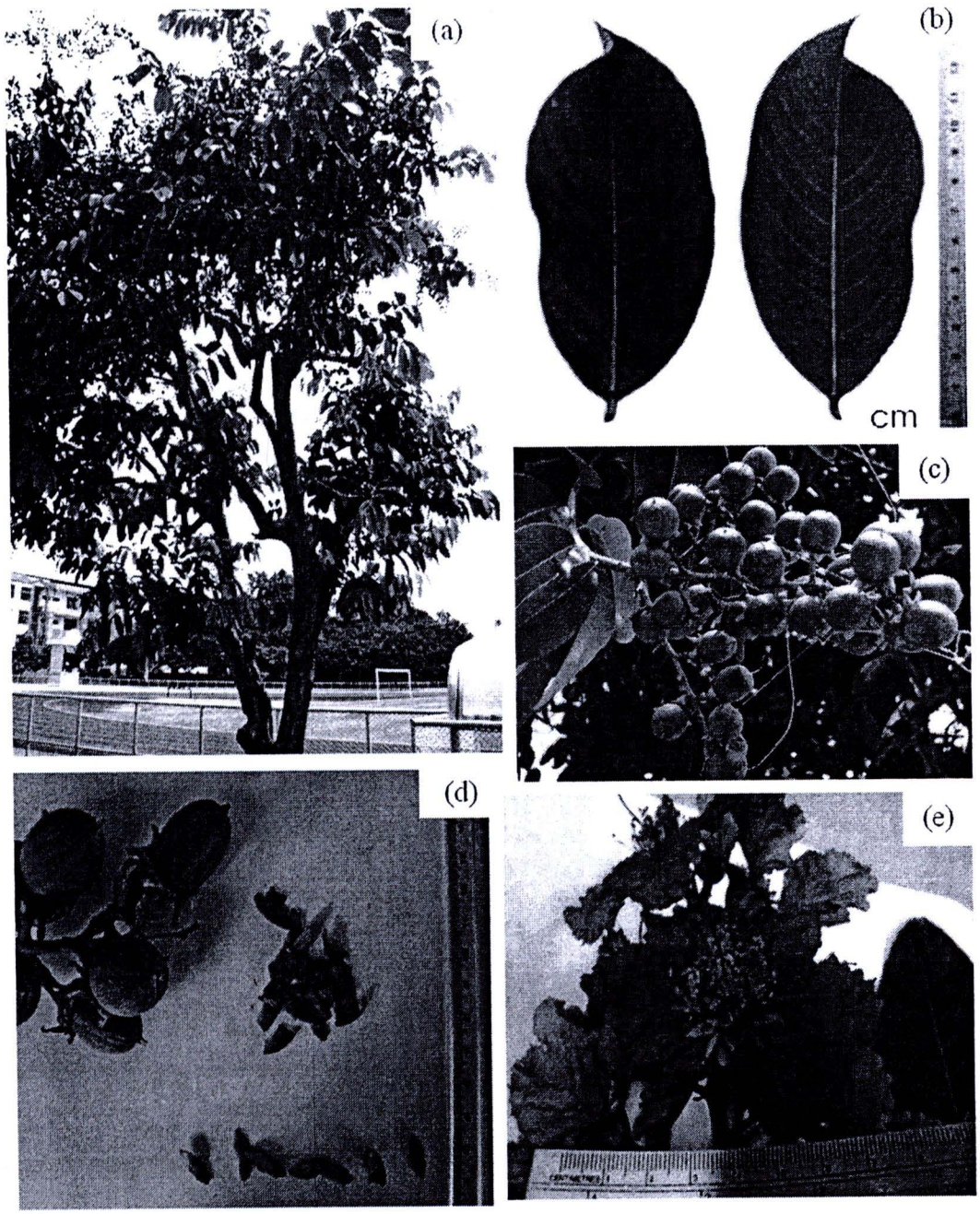

Figure 2.1 *Lagerstroemia speciosa* (L.) Pers. (a) habitat (b) leaves (c) infructescence (d) fruit and seeds (e) inflorescences.

Figure 2.2 Twig of *Lagerstroemia speciosa*, inflorescence and fruit.

2.2 Chemical constituents and biological activity of *L. speciosa*

The chemical constituents and biological activities of *L. speciosa* leaves are summarized in **Table 2.2**.

Table 2.2 The chemical constituents of *L.speciosa* leaves and its biological activity.

Chemical constituent	Category	Biological activity	Reference
Campesterol	Sterol	-	Sato *et al.*, 1981
Sitosterol	Sterol	-	Sato *et al.*, 1981
Stigmasterol	Sterol	-	Sato *et al.*, 1981
31-norargerenol acetate	Triterpene	-	Ragasa *et al.*, 2005
24-methylenecycloartanol	Triterpene	-	Ragasa *et al.*, 2005
Corosolic acid	Triterpene	Hypoglycemic activity and glucose transport activator	Fukushima *et al.*, 2006: Judy *et al.*, 2003; Kakuda *et al.*, 1996; Lui *et al.*, 2001; Miura *et al.*, 2004; Miura *et al.*, 2006; Murakami *et al.*, 1993; Shi *et al.*, 2008
		Anti-oxidant	Yamaguchi *et al.*, 2006
		Anti-inflammation	Yamaguchi *et al.*, 2006
		Anti-hypertension	Yamaguchi *et al.*, 2006
Cycloeucalenol acetate	Triterpene	-	Ragasa *et al.*, 2005

Chemical constituent	Category	Biological activity	Reference
Maslinic acid	Triterpene	-	Murakami *et al.*, 1993
Largerenol acetate	Triterpene	-	Ragasa *et al.*, 2005
3,3',4-tri-O-methylellagic acid	Tannin	Antioxidation	Takahashi *et al.*, 1977; Malaisree *et al.*, 2006
3-O-methylellagic acid	Tannin	Antioxidation	Takahashi *et al.*, 1977; Malaisree *et al.*, 2006
Ellagic acid	Tannin	Treatment of hyperuicemia	Sato *et al.*, 1990; Unno *et al.*, 2004
Flosin B	Tannin	Activator of glucose transport in fat cells,	Hayashi *et al.*, 2001
Lagerstroemin	Tannin	Activator of glucose transport in fat cells, Activated insulin receptor	Hattori *et al.*, 2003; Hayashi *et al.*, 2001
Reginin A	Tannin	Activator of glucose transport in fat cells,	Hayashi *et al.*, 2001
Valoneic acid dilactone	Tannin	Treatment of hyperuicemia	Unno *et al.*, 2004

Previous chemical investigations indicated that *L. speciosa* leaves contained terpenoids (Murakami *et al.*, 1993; Ragasa *et al.*, 2005), tannins (Tanaka *et al.*, 1992; Xu *et al.*, 1991[a]; Xu *et al.*, 1991[b]) and ellagic acids (Takahashi *et al.*, 1977). The corosolic acid was considered to be the active components responsible for the hypoglycemic activity of banaba (Hamamoto *et al.*, 1999; Lui *et al.*, 2005; Miura *et al.*, 2004; Murakami *et al.*, 1993).

Corosolic acid, a triterpenoid named 2α-hydroxyursolic acid (2α,3β-dihydroxyurs-12-en-28-oic acid), has been found in *L. speciosa* leaves. Recently, it has been reported to have antidiabetic activity in some animal experiments and clinical trials. Corosolic acid reduced the blood glucose levels and significantly lowered plasma insulin levels in KK-Ay mice 2 weeks after a single oral dose of 2 mg/kg. In addition, blood glucose in KK-Ay mice treated with corosolic acid significantly decreased in an insulin tolerance test (Miura *et al.*, 2006). The muscle GLUT4 translocation from low-density microsomal membrane to plasma membrane was significantly increased in the orally corosolic acid-treated mice when compared with that of the controls (Miura *et al.*, 2004). It has an effect on lowering postchallenge plasma glucose level *in vivo* in human (Fukushima *et al.*, 2006). Corosolic acid treatment subjects demonstrated lower glucose levels from 60 minutes until 120 minutes and reached statistical significance at 90 minutes (Judy *et al.*, 2003). So, corosolic acid was suggested to be a promising lead compound for diabetes treatment.

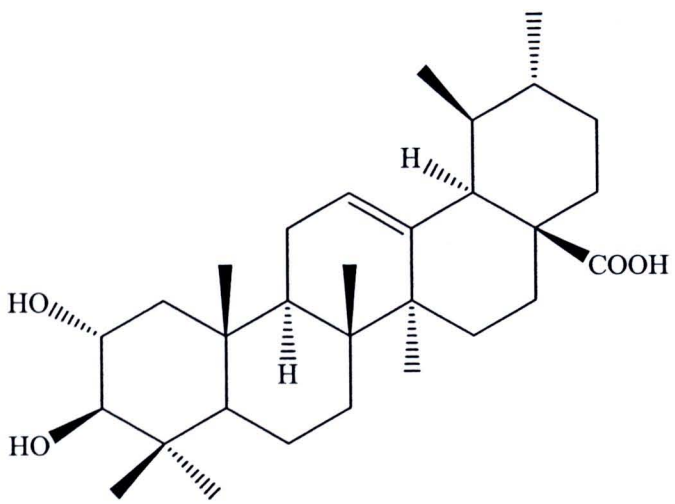

Figure 2.3 The chemical structure of corosolic acid.

2.3 Quality control methods for medicinal plant materials

Plant materials are used throughout developed and developing countries as home remedies, over-the-counter drug products and raw materials for the pharmaceutical industry, and represent a substantial proportion of the global drug market. It is essential to establish guidelines for assessing their quality. Thai Herbal Pharmacopoeia has emphasized the need to ensure the quality of medicinal plant products by using modern control techniques and applying suitable standards.

Pharmacognostic study is used to characterize and analyze the quality and quantity of crude drugs. In the macroscopic method, organoleptic sensation is used to determine the size, shape, color, odor, taste, *etc.* of the crude drugs, while the microscopic method revealed plant histological characteristics. The thin-layer chromatographic technique is used to differentiate extracts of different biological origins. Methods for quality control of crude drugs are described in Thai Herbal Pharmacopoeia (THP) and WHO guidelines.

2.3.1 Macroscopic examination

Medicinal plant materials are categorized according to sensory, macroscopic and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity and the degree of purify of such materials, and should be carried out before any further tests are undertaken. Wherever possible, authentic specimens of the material in question and samples of pharmacopoeia quality should be available to serve as a reference.

Visual inspection provides the simplest and quickest means by which to establish identity, purity and quality. If a sample is found to be significantly different, in terms of color, consistency, odor or taste, from the specifications, it is considered as not fulfilling the requirements. However, judgment must be exercised when considering odor and taste, owing to variability in assessment from person to person or by the same person at different times.

2.3.2 Inspection by microscopy

Macroscopic identity of medicinal plant materials is based on shape, size, color, surface characteristics, texture, and fracture characteristics. Microscopic inspection of medicinal plant materials is indispensable for the identification of broken or powdered materials. An examination by microscopy alone cannot provide complete identification, though when used in association with other analytical methods it can frequently supply invaluable supporting evidence. Microscopic measurements can be carried out using a stage micrometer in conjugation with an eyepiece micrometer and drawing adjustment.

The stage micrometer was used to measure the size of small objects. Two scales are required, known respectively as a stage micrometer and an eyepiece ocular micrometer. The stage micrometer is a glass slide 7.6 x 2.5 cm (3 x 1 inch) with a scale engraved on it. The scale is usually 1 mm long and is divided into 0.1 and 0.01 parts of a millimeter. The eyepiece micrometer may be a linear scale and the scale 0-10 or it may be ruled in squares. The value of one eyepiece division is determined for every optical combination to used, a note being made in each case of the objective eyepiece and length of raw-tube.

To calibrate micrometer, unscrew the upper lens of the eyepiece, place the eyepiece micrometer on the right inside, and replace the lens. Put the stage micrometer on the stage and focus it in the ordinary way, the two micrometer scales now appear (**Figure 2.4**), when the objective (x4) is in used. It will be seen that when the 7 line of the stage micrometer coincides with the 0 of the eyepiece, the 10 of the stage coincides with 7.7 of the eyepiece. As the distance between 7 and 10 on the stage scale is 0.3 mm, 77 of the small eyepiece division equal 0.3 m or 300 μm; therefore, 1 eyepiece division of this eyepiece micrometer which is used with the objective (x4) of this microscope equals 300/77 or 3.9 μm (Trease and Evans, 2009).

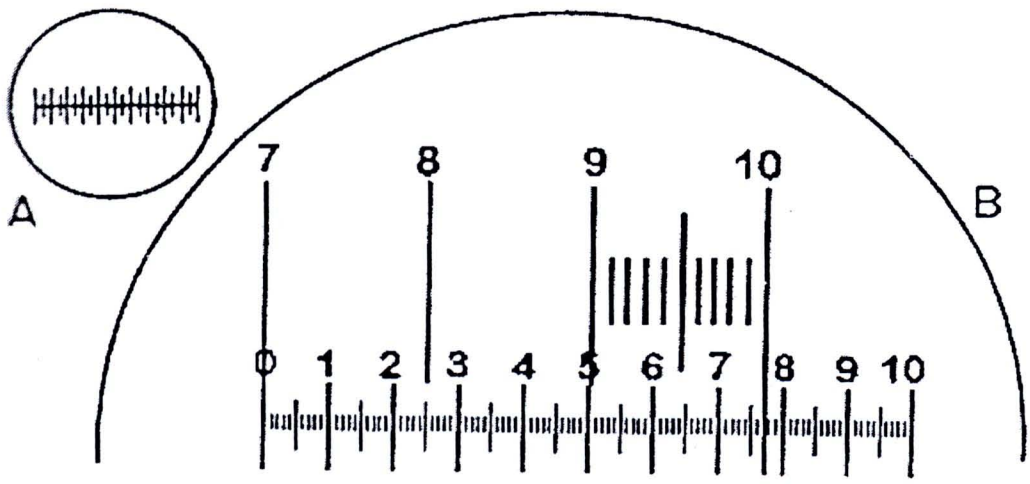

Figure 2.4 A. Eyepiece micrometer.

B. Eyepiece micrometer superimposed on portion of stage micrometer scale.

(Trease and Evans, 2009)

2.3.3 Constant values of leaves

A number of leaf measurements are used to distinguish between some closely related species not easily characterized by general microscopy.

2.3.3.1 Determination of stomatal index and stomatal number

Stomata are openings (the stomatal pores or apertures) epidermis bounded by two specialized epidermal cells, the guard cells, which by changes in shape result in the opening and closure of the aperture. It is convenient to apply the term stoma to the entire unit, the pore and two guard cells. The stoma may be surrounded by cells that do not differ from other guard cells of the epidermis. On the other hand, in many plants the stomata are flanked or surrounded by cells that differ in shape and sometimes also in content from the ordinary epidermal cells. These distinct cells are called subsidiary cell of the stoma. The subsidiary cells may or may not be closely related onto genetically to the guard cells. (Esau, 1960; Eames and MacDaniels, 1974)

In the mature leaves, four significant types of stomata are distinguished by their form and the arrangement of the surrounding cells, especially the subsidiary cells (WHO, 1998):

- The anomocytic or ranunculaceous (irregular-celled) type; the stoma is surrounded by a varying number of cells, generally not different from those of the epidermis (figure 2.5A).
- The anisocytic or cruciferous (unequal-celled) type; the stoma is usually surrounded by three or four subsidiary cells, one of which is markedly smaller than the others (figure 2.5B)
- The diacytic or caryophyllaceous (cross-celled) type; the stoma is accompanied by two subsidiary cells, the common wall of which is at right angles to the stoma (figure 2.5C).
- The paracytic or rubiaceous (parallel-celled) type; the stoma has two subsidiary cells, of which the long axes are parallel to the axis of the stoma (figure 2.5D).

In describing an epidermis where certain stomata differ from the predominant type, the term applying to the majority of stomata is used.

Stomatal number is the number of stomata per unit area of leaves. It was investigated by Timmerman in 1927 (Youngken, 1948). The actual number of stomata per square millimeter is variable for the same plant, this brings especially noticeable if records are made for different year (Wallis, 1960). The average number of stomata per square millimeter of epidermis is termed the stomata number. In recording result the range as well as the average value should be recorded for each surface of the leaf and the ratio of values for the two surfaces. In certain cases this ratio may be of diagnostic importance (Trease and Evans, 2009).

Stomatal Index is the percentage proportion of the ultimate divisions of the epidermis of a leaves which have been converted into stomata (Youngken, 1948).

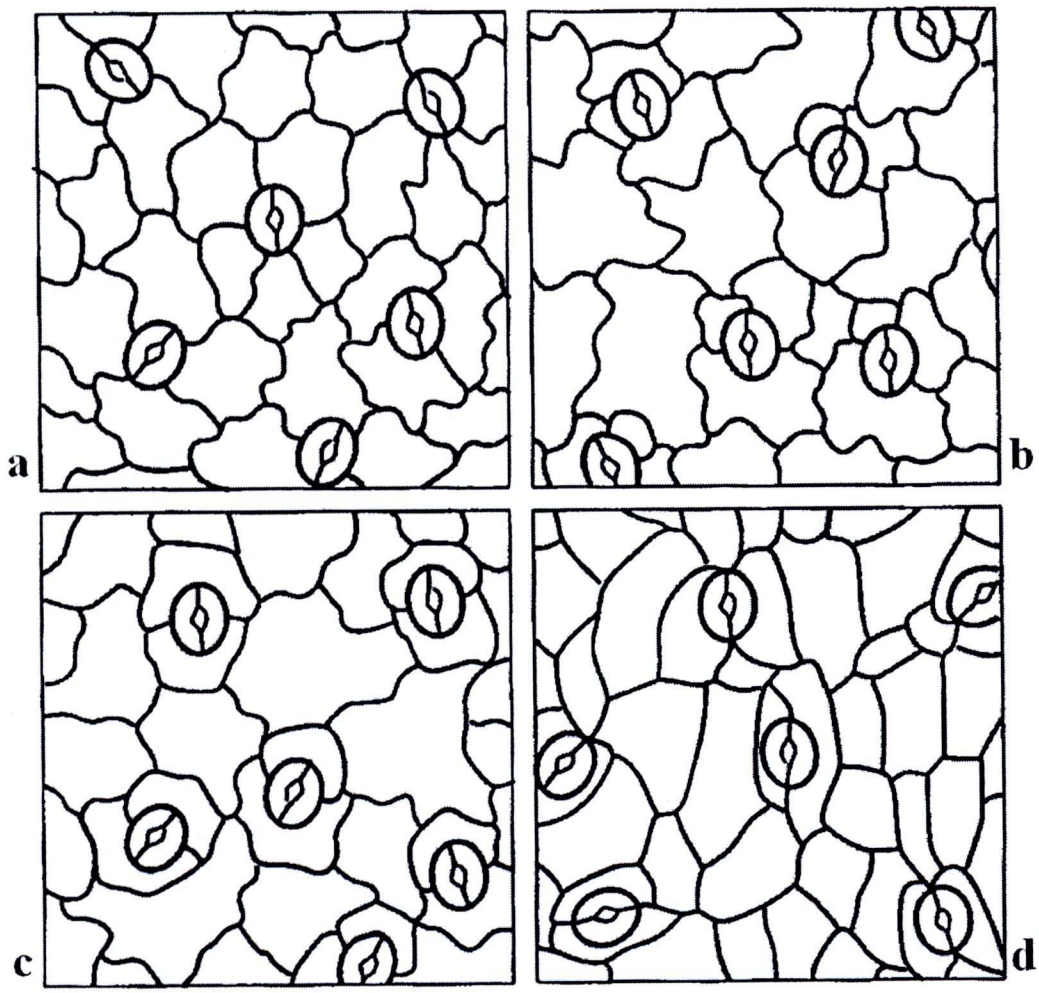

Figure 2.5 Epidermis in surface view illustrating patterns formed b guard cells and surrounding cells. a: anomocytic; b: anisocytic; c: diacytic; d: paracytic (WHO, 1998).

2.3.3.2 Palisade ratio examination

Palisade cells are a type of photosynthetic cells of the mesophyll of a leaf occurring mostly just beneath the upper epidermis surface layer (Esau, 1972; Wallis, 1960). The cells are elongated and more or less cylindrical and arranged in one or more rather regular, relatively compact layers near the ventral, or upper side of the leaf with the long axis of the cells perpendicular to the leaf surface (Eames and MacDaniels, 1974).

The term "palisade ratio" was introduced by two British pharmacognosists, T.E. Wallis and T. Dewar, in 1933. It represents a figure obtained by counting the total number of palisade cells beneath four upper epidermal cells and dividing the number by four (Youngken, 1948). The average number of palisade cells beneath each upper epidermal cell is termed the palisade ratio. Quite fine powders can be used for the determination (Trease and Evans, 2009).

2.3.3.3 Inspection of Vein-islet number

Vein-islets are divisions of green leaf tissue formed by the ultimate divisions of the conducting strands of vascular bundles which either completely or partially surround areas of the chlorenchyma. The islets increase in size as the leaf matures, the full grown leaf showing constancy in vein-islet number (Youngken, 1948). The term "vein-islet" is used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein-islet/mm^2 calculated from four contiguous square millimeters in the central part of the lamina, midway between the midrib and the margin, is termed the vein-islet number. When determined on whole leaves, the area examined should be form the central part of the lamina, midway between the margin and midrib (Trease and Eans, 2009).

2.3.3.4 Investigation of Veinlet termination number

Hall and Melville (1951) determined veinlet termination number. It means to the number of veinlet terminations per mm^2 of leaves surface. A vein termination is the ultimate free termination of a veinlet or branch of a veinlet. (Trease and Evans, 1996).

2.3.4 Thin-Layer Chromatography (TLC)

TLC, which together with paper chromatography comprises "planar" or "flatted" chromatography, is the simplest of all of the widely used chromatographic methods to perform. A suitable closed vessel-containing solvent and a coated plate are all that are required to carry out separations and qualitative and semi-quantitative analysis. With optimization of techniques and materials, highly efficient separations and accurate and precise quantification can be achieved. TLC can be used also for preparative scale separations by employing specialized apparatus and techniques.

Basic TLC is carried out as follows. An initial zone of mixture is placed near one end of the stationary phase, a thin layer; the sample is dried; and the end of the stationary phase with the initial zone is placed into a mobile phase, usually a mixture of pure solvents, inside a closed chamber. The components of the mixture migrate at different rates during movement of the mobile phase through the stationary phase, which is termed the development of the chromatogram. When the mobile phase has moved an appropriate distance, the stationary phase is removed, the mobile phase is rapidly dried, and the zones are detected by application of a suitable visualization reagent.

Differential migration is the result of varying degrees of affinity of the mixture components of the stationary and mobile phases. Different separation mechanisms are involved, the predominant forces depending on the exact nature of the two phases and the solutes. The interactions involved in determining chromatographic retention and selectivity include hydrogen bonding, electron-pair donor/electron pair acceptor (charge transfer), ion-ion, ion-dipole, and van der Waals interactions. Among the latter are dipole-dipole, dipole-induced dipole, and instantaneous dipole-induced dipole interactions.

Sample collection, preservation, and purification are problems common to TLC and all other chromatographic methods. For complex samples, the TLC development will usually not completely resolve the analyses (the substance to be determined) from interference unless a prior purification is carried out. This is most often done by selective extraction and column chromatography. In some cases, substances are converted, prior to TLC, to a derivative that is more suitable for separation, detection, and/or quantification than the parent compound.

Detection is most simple when the compounds of interest are naturally colored or fluorescent or absorb ultraviolet (UV) light. However, application of a

location or visualization reagent by spraying or dipping is usually required to produce color or fluorescence for most compounds. Absorption of UV light is common for many compounds, e.g., aromatics and those with conjugated double bonds. This leads to a simple, rather universal detection method on layers impregnated with a fluorescence indicator (fluorescence quench detection).

Compound identification in TLC is based initially on R_f values compared to authentic standards. R_f values are generally not exactly reproducible from laboratory to laboratory or even in different runs in the same laboratory, so they should be considered mainly as guides to relative migration distances and sequences. Factors causing R_f values to vary include: dimensions and type of the chamber, nature and size of the layer, direction of mobile phase flow, the volume and composition of the mobile phase, equilibration conditions, humidity, and sample preparation methods preceding chromatography (Sherma, 1991).

2.3.5 Phytochemical screening

The importance of plant-derived medicinal in modern medicines is often underestimated. A knowledge of the biological activities and/or chemical constituents of plants is desirable, not only for the discovery of new therapeutic agents but because such information may be of value in disclosing new sources of such economic materials. A knowledge of the chemical constituents of plants would further be valuable to those interested in the expanding area of chemotaxonomy (biochemical systematic), to those interested in biosynthesis, and to those interested in deciphering the actual value of folkloric remedies.

Phytochemical screening is the process of separation and isolation of active principle from plant sources. These screening are helpful to get the lead for discovery of the therapeutic agents.

The method for use in phytochemical screening should be (a) simple, (b) rapid, (c) designed for a minimum of equipment, (d) reasonably selective for the class of compounds under study, (e) quantitative in so far as having a knowledge of the lower limit of detection is concerned, and if possible, (f) should give additional information as to the presence or absence of specific members of the group being evaluated (Farnsworth, 1966)

2.3.6 Determination of physicochemical values

One possible problem in devising standards for crude drugs concerns the requirement for an assay of the active constituents when the latter may not have been precisely ascertained. Moreover, one of the principles of the herbal medicine is that the maximum effectiveness of the drug derives from the whole drug or its crude extract rather than from isolated components. In cases where an assay is lacking it is therefore of paramount importance that the crude drug is properly authenticated, its general quality verified and all formulations of it prepared in accordance with good manufacturing practice. Although official standards are necessary to control the quality of drugs their use doses raise certain problems. Of necessity, to accommodate is necessary to set relatively low standards which allow the use of commercial materials available in any season. There are a number of standards, numerical in nature, which can be applied to evaluation of crude drug either in the whole or the powdered condition (Trease and Evans, 2009). For this investigation, these are following:

2.3.6.1 Loss on drying

This is employed in the WHO guideline, EP, BP, USP and THP. Although the loss in weight, in the samples so tested, principally is due to water, small amounts of other volatile materials will also lead to the weight loss. For materials, which certain little balance combines the drying, process and weight recording; it is suitable where great numbers of samples are handled and where a continuous record of loss in weight with time is required (Trease and Evans, 2009).

2.3.6.2 Moisture content

The "loss on drying" methods can be made more specific for the determination of water by separating and evaluating the water obtained from a sample. This can be achieved by passing a dry inert gas through the heated sample and using an absorption train (specific for water) to collect the water carried forward; such method can be extremely accurate, as shown in their use for the determination of hydrogen in organic compounds by combustion analysis. Method based on distillation has been widely used for moisture determination. This method is employed in the WHO guideline, USP, BP and in THP and EP for some volatile oil-containing drugs (Trease and Evans, 2009).

2.3.6.3 Ash content

The determination of ash is useful to detecting low-grade products, exhausted drugs and excess of sandy or earthy matter; it is more especially applicable to powder drugs. Different types of ash figures are used such as total ash, acid-insoluble ash and water soluble ash. A total ash usually consists mainly of carbonates, phosphates, silicates and silica. If the total ash were treated with dilute hydrochloric acid, the percent of acid-insoluble ash may be determined. This usually consists mainly of silica and a high acid-insoluble ash in drug (Trease and Evans, 1996).

2.3.6.4 Extractive value

The determination of water-soluble or ethanol-soluble extractives is used as a means of evaluating drugs the constituents of which are not readily estimated by other means. In certain cases extraction of the drug is by maceration, in others by a continuous extraction process. For the latter the soxhlet extractor is particularly useful and has been in use for many years, not only for the determination of extractives (e.g. fixed oil in seed) but also for small-scale isolations (Trease and Evans, 2009).

2.4 DNA Fingerprinting

DNA technology provides a useful and independent tool to complement chemical analyses for the authentication and quality assurance of medicinal materials. DNA technology offers four advantages: (1) DNA-based markers are less affected by age and physiological conditions; (2) any part of the herb can be collected for analysis; (3) only a small sample is necessary for analysis; and (4) some DNA regions may be species-specific, whereas others may be family-specific. Benefiting of polymerase chain reaction (PCR) technique, DNA marker has now become a popular means for identification of herbal medicines.

The polymerase chain reaction (PCR) by Mullis *et al.* (1987) has opened a new approach for molecular genetic studies. PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Million copies of the target DNA sequence can be synthesized from a low amount of starting DNA template within a few hours. The PCR reaction components consist of genomic DNA used as DNA template for copies, a pair of primers for amplified in target sequences, dNTPs, buffer and *Taq* DNA polymerase. The amplification reaction consists of three steps; denaturation of dsDNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the anneal primers by heat-stable *Taq* DNA polymerase. The cycle is repeated for 20–40 times. Finally, the amplification products are examined by electrophoresis.

Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphisms. In terms of the mechanisms involved, DNA methods can be classified into three types, namely hybridization-based method, PCR-based method and sequencing-based method (Joshi *et al.*, 2004, Yip *et al.*, 2007)

Hybridization-based method

Restriction Fragment Length Polymorphism (RFLP) is the most widely used hybridization-based molecular marker (Semagn et al., 2006). The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. On an agarose gel, RFLP can be visualized using radiolabeled complementary DNA sequences. Polymorphisms are analyzed after hybridization by observing presence or absence of bands (Joshi *et al.*, 2004). The strength points of

RFLP markers are high reproducibility, codominant inheritance, no sequence information required, and relatively easy to score due to large size difference between fragments (Semagn *et al.*, 2006). There are, however, some problems with the RFLP method of DNA fingerprinting. Firstly, the results do not specifically indicate the chance of a match between two organisms. Secondly, the process involves a lot of money and labor, which not many laboratories can afford (Vasudevan, 2007).

DNA sequencing-based method

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined regions. There are two general methods for sequencing of DNA segments: the "chemical cleavage" procedure (Maxam and Gilbert, 1977) and the "chain termination" procedure (Sanger, 1977). Nevertheless, the latter method is more popular because chemical cleavage method requires the use of several hazardous substances. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library.

Nowadays, the sequencing method developed by Fred Sanger forms the basis of automated "cycle" sequencing reactions. DNA sequencing reaction is like the PCR reaction for replicating DNA. The reaction mix contains template DNA, primers, DNA polymerase, four nucleotides (dGTP, dCTP, dATP and dTTP). In addition, a second type of nucleotide called dideoxynucleotide, are added in the reaction mix. These dideoxynucleotide are labeled with fluorescent dye and can be recognized by DNA sequencer. To start the reaction, the mixture is heated until the two stands of DNA separated. Then the primer sticks to its intended location and DNA polymerase starts elongating the primer. If allowed to go to completion, a new strand of DNA would be the result. The enzyme makes no difference between dNTPs or ddNTPs. When a ddNTP is included, the synthesis stops. Because billions of DNA molecules are present in the tube, the stand can be terminated at any position, so different lengths of DNA stands are emerged. Then, the reaction is transferred to polyacrylamide gel. The gel is placed into a DNA sequencer for electrophoresis and analysis. The fragments migrate according to size, and each is detected as it passes a laser beam at the bottom of gel. Each dideoxynucleotide emits colored light of a

characteristic wavelength and is recorded as a colored band on a simulated gel image (Weising *et al.*, 2005)

DNA sequencing is the most optimal method for population genetic applications particular phylogenetic studies of organisms. This technique provides high resolution and facilitating interpretation. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The sequencing method has been facilitated by the direct and indirect use of DNA fragments generated through PCR. At present, automatic DNA sequencing has been introduced and commonly used. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

PCR-based method

PCR-based methods use amplification of particular DNA sequences or loci, with the oligonucleotide primers and the thermostable DNA polymerase enzymes (Joshi *et al.*, 2004). PCR-based methods include PCR-restriction fragment length polymorphism (PCR-RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single strand conformation polymorphism (SSCP), sequence characterized amplified regions (SCAR), amplification refractory mutation system (ARMS), simple sequence repeat (SSR) analysis, direct amplification of length polymorphism (DALP), inter-simple sequence repeat (ISSR), directed amplification of minisatellite-region DNA (DAMD) (Yip *et al.*, 2007)

Amplified Fragment Length Polymorphisms (AFLPs) technique, a PCR-based molecular marker, was first developed by Vos and Zabeau, researchers of Keygene N.V. company in Netherlands, and registered a patent in 1993 (Vos *et al.*, 1995). AFLPs analysis was clearly a powerful technique in terms of its ability to identify a large number of polymorphic bands without any prior knowledge of the organisms. The ability of this technique to generate many markers with minimum primer testing and the system's high resolution (i.e., band clarity and relative low lane background) are features that make AFLP attractive as genetic markers (Crouch *et al.*, 1999). Moreover, AFLP analysis is used in variety of fields, including plants, animal breeding, medical diagnostics, forensic analysis, microbial typing, *etc.*

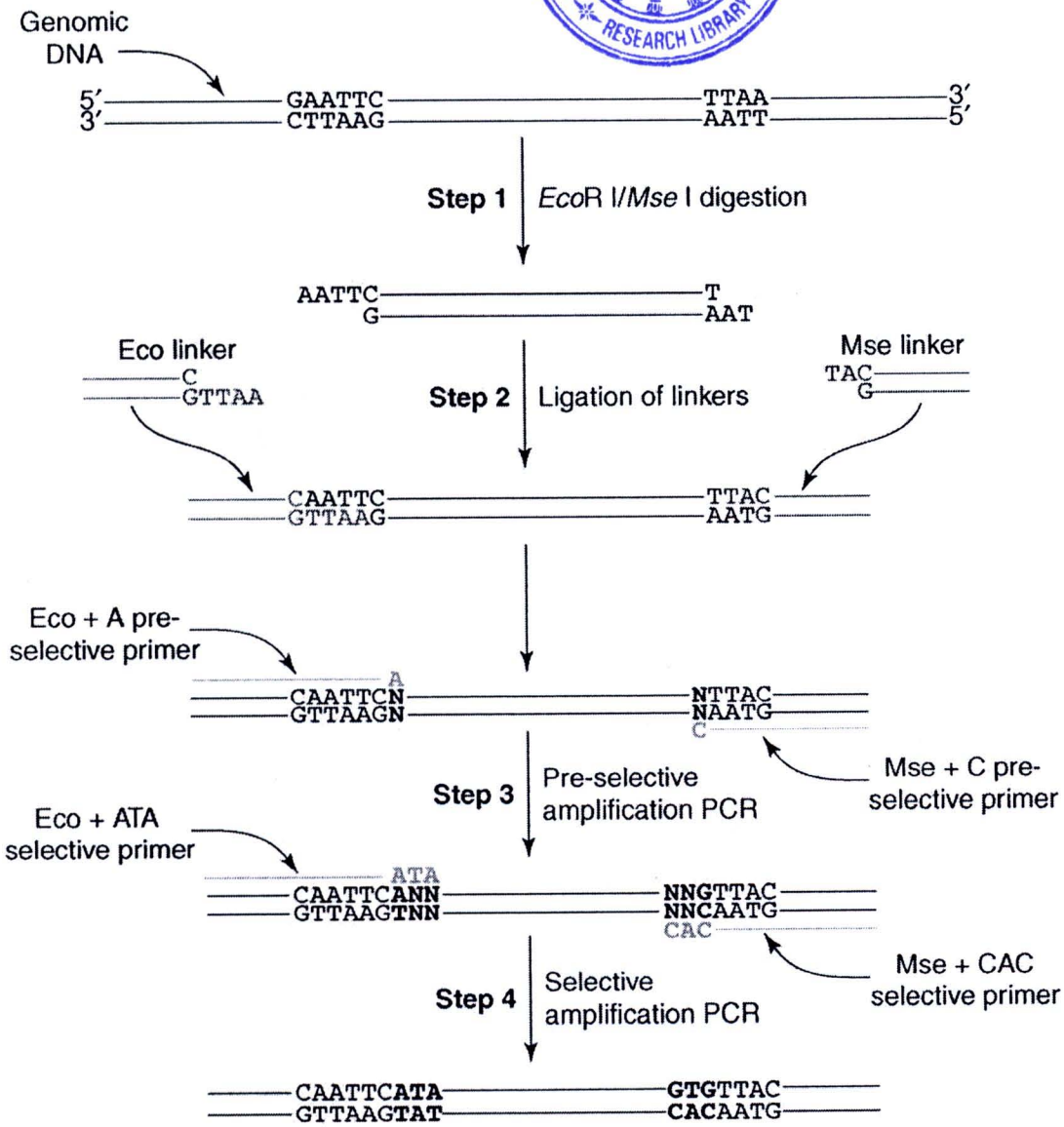

Figure 2.6 The process of Amplified Fragment Length Polymorphism (AFLP) technique. (Meudt *et al.*, 2007)

AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al.*, 1995). The technique combined the reliability of the Restriction Fragment Length Polymorphism (RFLP) analysis with the power of the PCR technique. The technique involves three steps (**Figure 2.6**). The first step is restriction endonuclease digestion of genomic DNA with a restriction enzyme that cuts frequently (*Mse*I, 4 bps recognition sequence) and the other that cut less frequently (*EcoR*I, 6 bps recognition sequence). The following step is ligation of double stranded adapters to ends of the restriction fragments. The frequent cutter is used to generate small fragments. It is amplified in the optimal size range for separation on a polyacrylamide gel. The rare cutter is used to position the attachment of two primers in the next PCR amplification. Selective amplification of sets of restriction fragments using selective AFLP primers is performed. This step increases the number of fragments by selective primers which are the same base sequence with adapters. The PCR amplification uses primer to add nucleotides at 3′ ends, which adds two more nucleotides with two steps selective amplification. The first step is selective amplification using three nucleotides containing further additional primer bases. This step uses selective amplification of sets of restriction fragments and reduces the number of fragments because a single 3′-extension on both primers reduce the number of fragments amplified by a factor of 16, a two-base extension reduce the number by 256, and a three-base extension reduces it by 4,096. The last step, analysis of the amplified fragments on polyacrylamide gels and DNA band visualization is generated.

AFLPs marker is extensively used for studying genetic diversity in different plant species (Steiger *et al.*, 2002; Vos *et al.*, 1995). Comparative studies using restriction RFLPs, RAPD, AFLP, and microsatellite techniques have shown that AFLP method is the most efficient method to estimate genetic diversity because of its high reproducibility, high quantity of information throughout multiple loci on the genome, high resolution enough to determine some small genetic differences and generate multiplex ratio of data for numerical analysis. RAPD markers have the high variable of replicability, but easy to use. RFLP technique provides low quantity of information, but has higher replicability and resolution of genetic differences when compared to RAPD technique, but lower than AFLP and microsatellite techniques. Microsattellite method has good qualification as well as AFLP, but needs some

knowledge about genetic information which takes development time, difficult to use and develop the process. However, AFLP and microsatellite techniques, coupled with sequencing information for systematic analyses, could be synergized as main tools for the analysis of genetic variation (Mueller and Wolfenbarger, 1999).

AFLPs technique is being widely used for genetic diversity studies because it shows significantly polymorphism and is robust and reliable for molecular genetic marker assay. AFLP fingerprinting analysis has been used to discriminate between accessions of a number of plants *i.e.* olive (Angiolillo *et al.*, 1999), jackfruit (Schnell *et al.*, 2001), Neem; *Azadirachta indica* (Singh *et al.*,2002), *Erythroxylum* (Johnson *et al.*, 2003), *Prunus* (Aradhya *et al.*, 2004), *Gardenia jasminoides* (Han *et al.*, 2007), *Boesenbergia* (Techaprasan *et al.*, 2008), pomegranate (Jbir *et al.*, 2008), *Panax japonicus* (Choi *et al.*, 2008) Tunisian Fig; *Ficus carica* (Baraket *et al.*, 2009), *Fritillaria cirrhosa* (Zhang *et al.*, 2010).