The Breeding System of Kacangma and the Effect of Drying Processes on the Leaf’s Flavonoids Content

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The Breeding System of Kacangma and the Effect of Drying Processes on the Leaf’s Flavonoids Content

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This project is submitted in partial fulfillment of the requirement for degree of Bachelor of Science with Honours

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I declare that no portion of this research work had been submitted to support the application of other degree or qualification at any other universities or institutions of higher learning.

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<tr>
<td>AA</td>
<td>Acetic acid (15%)</td>
</tr>
<tr>
<td>BAW</td>
<td>n-Butanol: Acetic acid: Water</td>
</tr>
<tr>
<td>LS</td>
<td>Leonurus sibiricus</td>
</tr>
<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Retardation factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
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<td>UV (B)</td>
<td>Ultraviolet (B)</td>
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<td>2-D paper chromatography</td>
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The Floral Development of Kacangma and the Effect of Drying Processes on the Leaf’s Flavonoids Content

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ABSTRACT

Leonurus sibiricus is an annual herb that belongs to the family Lamiaceae. In Malaysia, there are two species found, namely Leonurus sibiricus L. (pink-flowered variety) and Leonurus japonicus Houtt (white-floured variety). Both of these species are easy to distinguish by colour, however, both of them do not differ much based on their vegetative characteristics and also their phytochemical content. Pollen happens to be an important vector of gene flow in plants. In the current study, it showed that the pollen viability might affect greatly due to the concentrations of culture media (agar) used and the duration of incubation. Both of these conditions exhibited negative correlation with the viability tests done on L. sibiricus. In the phytochemical analysis, the current study showed that the flavonoids compounds in L. sibiricus were growth stage dependent, hence the medicinal values may differ based on their growth stages. In addition, the current study also showed the effects of different drying methods on the L. sibiricus leaves. Sun-dried leaves exhibited more compound contents and antioxidant capacity compared to air-dried leaves, whereas the exposure of oven-drying on the L. sibiricus leaves have caused antioxidant loss due to the heat instability compounds.

Keywords: drying quality, flavonoids contents, growth stages, Leonurus japonicus, Leonurus sibiricus L., phytochemical analysis, pollen viability

ABSTRAK


Keywords: Leonurus sibiricus L., Leonurus japonicus, daya maju debunga, analisis fitokimia, kandungan flavonoids, peringkat pertumbuhan, kualiti jemuran
CHAPTER 1
INTRODUCTION

*Leonurus sibiricus* L. is an annual herbaceous species from the family Lamiaceae. It is most commonly known as motherwort, kacangma (in Malay) and I-mu-ts’ao (in Mandarin). *L. sibiricus* originate from North East of Asia and Japan, however it is also found in countries such as China, Manchuria, Siberia, Indochina, India, Africa, America (Keys, 1991) and Malaysia (Burkill, 1966).

In Malaysia, *L. sibiricus* was brought into the country for cultivation. There is another species of kacangma in Malaysia, *L. japonicus* Houtt (white flowered) (Hu, 1976 and Govaerts, 2003). The two species are difficult to be distinguished based on their vegetative characters but can be clearly differentiated by the colour of flower.

Kacangma is a popular traditional herb that has been consumed for decades by the people of Sarawak as an herbal medicine or culinary ingredient (Chai et al. 1989; Teo and Chua, 2001). The role for this particular species as an underutilized herb with potential economic value has been recognized (MOA 1995; Paulus and Lau, 2004). Subsequently, efforts are made to increase the utilization by developing kacangma herb into various special products with commercial significance. Lau (2003) has reported that *L. sibiricus* has been commercialized locally into dried and chopped kacangma leaves/stem, ready packed products, as well as canned kacangma meat products. For medicinal purposes, *L. sibiricus* is preferred by the Sarawak local community, especially the Chinese. It is claimed that this *L. sibiricus* is more potent medicinally as compared to *L. japonicus* (Per. comm. Lau C. Y., Agriculture Research Centre, Semonggok). Hu (1976) stated that these two species have their own unique medicinal value in which *L. sibiricus* is good for blood
enrichment while *L. japonicus* is good for stamina improvement.

There were limited numbers of studies regarding *L. sibiricus* which focuses on the study of pollination biology. Based on the studies made by Yeo (2005), he reported that both of the varieties are self-pollinated, in which the pollination occurs about or before anthesis happens. In relation to the findings done by Yeo (2005), the determination of the pollen viability is essential in improving the efficiency of a breeding program and increasing the success of the artificial pollination conducted (Bolat & Pirlak, 1990; Pline et al., 2002). Pollen viability is generally considered to indicate the ability of the pollen grain to perform its function of delivering the sperm cells to the embryo sac following the compatible pollination (Shivanna et al., 1991). The observation is done on different agar concentrations at different duration of incubation, to determine at which conditions that produces the most viable pollen. Knowledge about the duration of pollen viability will be helpful in developing various methods to manage pollen flow.

Previous research showed that *L. sibiricus* and *L. japonicus* have the same basic phytochemical content (Newall et al. 1996). Flavonoids are one of the phytochemicals present in kacangma. Flavonoids are natural phenolic compounds which are widely used in chemotaxonomy. The occurrence of flavonoid is not restricted only to flower but also includes all part of plant and can be easily detected. It is also known to be inherited codominantly (Talukdar, 2012). Flavonoids are potential as marker in breeding. Plant growth stages, however, may influence the production of phytochemicals (secondary metabolites) (Wang S.Y. et al., 2009). Flavonoids content in *L. sibiricus* are differed at different growth stages, however, is based on non-repeated expereiment (Najihah, 2014). This will affect the application of flavonoids as marker for breeding.
According to Chua & Aminah (2013), the \textit{L. sibiricus} traditionally is never consumed raw due to its strong herbal scent. It is usually processed into dried leaves before utilizing it as culinary ingredient. For commercial purposes, the kacangma leaves/stems are chopped and then undergo drying process, in order to prolong the storage life of the herb. Although reports are limited, studies indicate that drying conditions can impact the chemical and biological activities of herbs (Capecka et al., 2005; Lim and Murtijaya, 2007). Nonetheless, it is also not uncommon for drying conditions to increase the chemical and biological content of herbs. Drying must be performed carefully in order to preserve the aroma, appearance and nutritional characteristics of the raw herbs as much as possible (Crivelli et al., 2002). Thus, this study will provide information on which suitable drying conditions that sustains most of antioxidant properties, and put both species in use by many local folks for their medicinal value.

This thesis focused on the pollen viability of \textit{L. sibiricus}, in which parameters such as the different concentrations of agar used in the culture media and the durations of pollen incubation were measured, to evaluate the optimized condition suitable for pollen germination of this species. Furthermore, it also aimed to analyze flavonoids as potential markers and evaluate the effects of different drying methods on the \textit{L.sibiricus} leaves.

Therefore, the aim of this study was to cover the aspects of pollination biology and provide important data for the development of this herb. The information obtained from this study could be useful for other closely related species.
1.1 **Problem Statements**

i. There are limited number of studies regarding the study of pollen viability of *Leonurus* species.

ii. The flavonoids polymorphism between the *Leonurus* species at different growth stages reported by Najihah (2013) is based on non-repeated experiment.

iii. Limited studies on the impact of drying conditions on the phytochemical content of *Leonurus* species.

1.2 **Objectives**

i. To study the pollen viability of *Leonurus sibiricus* with the influences of different variables: the concentration of agar used in culture media and the duration of incubation of the pollens.

ii. To determine the difference of flavonoid contents at different growth stages of kacangma.

iii. To study the effects of different drying process on the flavonoids content of kacangma.
CHAPTER 2
LITERATURE REVIEW

2.1 Botanical description and taxonomic classification

The leaf morphology of *Leonurus sibiricus* (pink-flowered variety) are naturally producing different types of leaves as the plant matures. The lower ones are broadly ovate to deltate in outline, deeply palmate lobed, while the upper leaves gradually reduced in size and with few smaller lobes (Globinmed). Based on the observation by Hu (1976), the inflorescence of the *L. sibiricus* is verticil. Verticillasters has about 8-15 flowered, sub-sessile flower leaves, and short, spiny bracteoles (http://www.efloras.org). The flowers are crowded and occur in numerous whorls (Wei, 2014). Hu (1976) also reported that the corolla of the flower is bilabiate and it has a woolly features on its outer part. The androecium has two pairs of exerted stamens, while the gynoecium has a deeply cleft ovary with a long-thin style and a bifid stigma.

The white-flowered variety, known as *Leonurus sibiricus var. albiflorus* Migo has a similar vegetative characteristics as to *L. sibiricus*, but can be clearly differentiated based on the colour of flower. The synonym for this species is *Leonurus japonicus* Houtt, in which this record derives from the World Checklist of Selected Plant Families (WCSP) on March 2012, which reports it as a synonym (www.theplantlist.org/).

2.2 Ethnobotanical data

*Leonurus sibiricus* L. (kacangma) is a popular traditional herb and is widely used in Sarawak. According to Chua & Aminah (2013), kacangma traditionally is never consumed raw due to its strong herbal scent. It is usually processed into dried leaves before
utilizing it as culinary ingredient, such as kacangma soup. For commercial purposes, the kacangma leaves/stems are chopped and then undergo drying process, in order to prolong the storage life of the herb (Chua & Aminah, 2013).

In traditional medicine, the whole part of *Leonurus* plant, including the flowers and seeds are used. According to Duke and Ayensu (1985), the whole parts of the *Leonurus* plant has different function. The seeds are used to treat diuretic, aphrodisiac and emmenagogue. The dried flowers help remove placenta after birth, while the juice from the stem is used to treat wounds. Besides that, Wei (2014) also reported that the *Leonurus* plant is used to treat post-partum haemorrhages and menstrual disorders.

Hu (1976) also claimed that in the Chinese community, *L. sibiricus* is able to improve vision, dehydration, fever, stimulate blood circulation, strengthen liver, reduce cardiovascular illness, cure diseases associated with child birth, etc.. Besides medicinal use, it is also used as tonic (Muhamad & Mustafa, 1992). Externally, the *Leonurus* herbs can also be used in cosmetics (Hu, 1976; Tanaka *et al*., 2001). Tanaka *et al*. (2001) reported that the extract from the *Leonurus* herbs helps stimulate the superoxidant dismutase activity and protects the skin from harmful ultraviolet B (UVB) rays.

### 2.3 General constituents of *L. sibiricus*

*Leonurus sibiricus* generally constitutes of essential oils, alkaloids (stachydrine, leonurine), glycosides (lenurine, leonuridin), flavonoids, diterpenes, caffeic acid tannins, and vitamin A (Lau, 2003). The ethanol extract from the aerial part of *L. sibiricus* is cytotoxicity positive against leukaemia cells (L 1210). The ethanol extract consists of two new furanoditerpenelactones, leonotinin, leonotin, Dublin and nepetaefuran. All of these
six compounds showed moderate cytotoxic activity against leukaemia cells (L 1210) in tissue culture (Satoh et. al., 2003).

One same plant species may have different composition of nutrients according to intrinsic factor (genotype, variety, maturity stage and parts of plants) and extrinsic factor (light intensity, temperature, water and realibility nutrients) (Bernath, 1986). The two varieties of \textit{L. sibiricus} both have different content of nutrient that depends on their stage of maturity. The crude fibre and carbohydrate contents of \textit{L. sibiricus} increased with the plant maturity while its moisture, protein and ash contents declined. The content of fat in \textit{Leonurus} will decrease on day 70\textsuperscript{th} but increase on the 100\textsuperscript{th} day (Chua & Aminah, 2003). The drop value of fat is due to the formation of nectar gland and also because of collection of variety type polysaccharide sugar (Cao et al., 2000).

Both \textit{L. sibiricus} and \textit{L. japonicus} contain iron but the value of iron in both species differs according to the maturity stage. The iron content in \textit{L. japonicus} increases constantly as it matures while \textit{L. sibiricus} has constant value of iron and no significant difference on iron content as it matures (Chua & Aminah, 2003). Whereas for vitamin A, B2 and C, they declined with maturity but there is no significant difference in the B1 content.

In general, \textit{L. sibiricus} contained higher level of nutrients compared to \textit{L. japonicus} except for vitamin A. The nutrients contents of \textit{L. sibiricus} are adversely affected by weather and high rainfall (Chua & Aminah, 2003).
2.4 Chemotaxonomic study

Chemotaxonomy is a study of classifying and identifying organisms or plants according to demonstrable differences and similarities in their chemical constituent (Lai et al., 2001).

Flavonoids has been utilized in the chemotaxonomic study for the Lamiaceae at various taxon (Tomas-Barberan & Gill, 1992; cited by Upson et al., 1999). However, there is no report on the study of *L. sibiricus*. Flavonoids are ideal molecules for chemotaxonomic study due to its structure diversity, widespread occurrence, stability and ease of identification (Bohm, 1998). Not all plant groups have same classes of flavonoids. If there are plants that possess the same flavonoid classes, the individual compounds would not be the same in all species within the groups (Bohm, 1998).

There is several importance of utilizing flavonoids in chemotaxonomic study. Flavonoids can be one of the solutions in plant identification when flowering and fruit development does not occur frequently (Joshi, 2003). It is also useful in solving the classification of genera such as the taxonomy of *Cotylelobium* species (Joshi, 2008). Flavonoids had also resolved the genus *Erythroxylum* based on the uniqueness or polymorphisms of leaf flavonoids (Johnson et al., 1998, 2002, 2003; Johnson & Schmidt, 1999).

Phytochemical marker can also be used to resolve the controversies about phylogeny and affinity of infra-familial groups as well as uncertain positioning of some genera such as Asteraceae (Emerenciano et al., 2001).

The leaf flavonoids are proven to be a valuable tool in determining the origin of hybrids (Williams et al., 1983). Flavonoid chemistry had been used in recognition of the natural
hybrids of species *Podocarpus* also in conjunction with morphological characters and geographical considerations (Rosemary *et al*., 1987). Voirin *et al.* (1990) on the other hand had used the flavonoid aglycones as markers of parentage in *Mentha aquatic, M. citrate, M. soicata and M. x piperita* which the occurrence of hybridisation is frequent either from natural origin or in cultivation.

2.5 Cultivation

*L. sibiricus* is propagated by seed, which when lightly covered in soil and moistened-well, it will germinate and grow quickly in full sunlight. Although this type of plant is able to survive under severe conditions, Lau (2003) claimed that this herb prefers moist and well-drained soils. Hu (1976) reported that the *L. sibiricus* plant can reach up to one meter in height, with numerous branches when planted with fertile soil. However, under poor soil condition, the growth of the herb will appear stunted and without branches. It is also sun-loving and can thrive under partly shaded places. Besides that, *L. sibiricus* is resistant to drought. It grows well both in full garden beds and in pots (USDA n.d., ?).

The study on planting kacangma commercially in Sarawak was done by Lau (2003) and Tawan & Sim (2005). Field planting of *L. sibiricus*, first requires raised planting beds. Then, basal dressings of 2 kg/m² of well decomposed chicken manure and 50 g/m² of compound fertiliser (15: 15: 15) are applied and rotovate lightly planting beds to mix well fertilisers into top 20 cm of beds. Two g/m² of seeds are sown. Sowing can be done all-year round, except on November until January because the *L. sibiricus* cannot germinate with damp soil condition. The beds are covered with black sheer material after spreading the seeds and allow the seeds germinate for 10 to 15 days. Snail baits are used to avoid
snail damage of seedlings. After one month of sowing, a round of compound fertiliser (15:15:15) at 50 g/m² is required.

Besides snail damage, there is no other major pest or disease reported attacking this particular plant (Lau (2003), cited by Yeo, 2005). Lau (2003) also claimed that, organic production of *L. sibiricus* is possible.

### 2.6 Pollination

Dafni (1992) has recommended several important methods on cross and self-pollination test. Firstly, isolation bags or fine mesh are used for covering the plant or flower before flowering event. Secondly, flower that had opened needs to be removed. Before any pollen is produced, the selected flower is emasculated, and the plant or flower is recovered. Next, artificial pollination is done to the un-masculated and emasculated flowers, and then any pollinated flowers are bagged again.

### 2.7 Pollen viability test

Pollen viability test is a method to get an indication of the ability of the pollen grains to perform the function of delivering the sperm cell to the emryo sac during pollination (Shivanna *et al.*, 1991). There are several methods that can be used for evaluation of pollen viability, for example using staining method, in vitro germination, in vivo germination and artificial crossings. Based on the reports done by Dafni & Firmage (2000), the methods used depends on the crop being investigated. There are currently limited information on which methods are the best for different crops. By using information from different tests that have been carried out on other crops may help in refining the specific methods for specific crops.
In vitro germination for pollen viability is where fresh harvested pollen is grown on medium containing sucrose, boric acid and calcium nitrate in a humid environment and at room temperature (~20°C) (Wang et al., 2004). Fang et al. (2013) stated that pollen was view germinated when the length of the pollen tube exceeded the diameter of its pollen grain. Pollen viability was calculated as the ratio of the number of germinated pollen grains to the total number of pollen grains (Fang et al., 2013). The in vitro test is more widely used than other tests due to its rapidness and easiness of the test. The most frequently used media is the modification of the Brewbaker and Kwack (1964) media (B-K media) which consists of 1 – 4 mM boric acid, \( \text{H}_3\text{BO}_3 \) and 1 – 4 mM calcium nitrite, \( \text{Ca(NO}_3\text{)}_2 \) in 0.30 to 0.90 M (10 -3% sucrose) (Heslop-Harrison, 1984; Pline et al., 2002).

The knowledge about the duration of pollen viability will also be helpful in developing various methods to manage pollen flow. From the studies done on tall fescue plant by Wang et al. (2004), the pollen were maintained under controlled conditions in growth chamber at various time points and it was found that the viability decreased gradually with time. Viability of pollen grain can last as long as 22 hours under the controlled conditions, however there were fewer data to support this (Wang et al., 2004).

2.8 Medical study

Kaefer and Milner (2008) stated that dietary intake of herbs and spices with high antioxidant contents may improve our resistance to free radical damage and thus risk of a heart disease and cancer. Studies show that herbs generally contain higher level of antioxidant content than fruits, vegetables and nuts (Konczak et al., 2010; Zheng & Wang,
Leonurus had been proved for having antioxidant activity which can prevent oxidation of lipid thus may overcome rancidity of food product (Chua & Aminah, 2010).

However, large consumption of L. sibiricus may give high toxicities which can lead to internal organ damage. Studies on the toxicity of L. sibiricus evaluated in acute and sub-chronic studies on New Zealand male and female rabbits shows that there is no toxicity was seen on consumption at rate of 0.5 g/kbw (low dose) on a 90-day sub-chronic study. However, there are some indications of renal and liver toxicities were noted in the medium and high dose groups (Chua et al, 2008).

Herbs such as L. sibiricus have been used not only as food preservatives and flavouring, but also as traditional medicines for thousands of years. In traditional Chinese medicine, the herb is used to treat loss of potency in men, postpartum bleeding or painful menstruation in women, and as a diuretic (Keng, 1974). Shi et al. (1995) claimed that the decoction of Chinese drugs L. sibiricus being positive in having action on the uterus of mouse in vitro. Based on clinical test, Leonurus herbs have sedative effect, antihypertensive and uterotonic. They also had been used as body pain reliever and emmenagogue for post-natal care (Chai et al., 1989).

Further study on the anti-tumour effects of L. sibiricus medicinally was done by Nagasawa et al. (1992). Nagasawa et al. (1992) had studied the effects of L. sibiricus on the restoration of lactation suppressed by pregnancy-dependent mammary tumours (PDMT) in GR/A mice. According to the study, the L. sibiricus were able to ameliorate lactation suppressed by PDMT through stimulation of both growth and function of mammary gland. Also, L. sibiricus had elevated the mammary DNA and RNA contents in PDMT mice.
CHAPTER 3
MATERIALS AND METHODS

3.1 Sowing

The seeds of *Leonurus sibiricus* were germinated on tray containing sand and organic compost at a ratio of 1:1. When the seeds are germinated, two to four weeks old seedlings were transplanted to the plant beds prepared from a mixture of top soil, compost and sand with ratio 3: 2: 1, at the researcher’s backyard in Lundu. Then, the plant beds were covered with black sheer material to provide some shade to the newly transplanted seedlings.

3.2 Pollen Viability Test

Fresh pollens are used for this test in which unopened flowers of *L. sibiricus* were harvested to have enough pollen for the germination series.

3.2.1 Media Preparation

The pollen viability test was conducted using a mixture of agar powder, glucose powder and distilled water. This test was conducted on three different agar concentrations which were at 12.5% concentration, 6.25% concentration and 2.5% concentration. The mixtures of each concentration were shown in Table 3.2(a).

<table>
<thead>
<tr>
<th>Agar Concentrations (%)</th>
<th>Agar Powder (gram)</th>
<th>Glucose Powder (gram)</th>
<th>Distilled Water (ml)</th>
</tr>
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<tbody>
<tr>
<td>12.5</td>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>6.25</td>
<td>7.5</td>
<td>60</td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
<td></td>
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</table>
3.2.2 *In vitro* Germination Test

The germination test involved were to study on two parameters: the effects of different agar concentrations on the growth of pollen and the effects of duration of incubation on the rate of pollen germination.

The pollen collected were spread across the agar media prepared previously. The petri dishes containing the pollens were incubated at 20°C at various time points: one hour, three hours, six hours and 24 hours (Table 3.2(b)).

<table>
<thead>
<tr>
<th>Table 3.2(b) : Pollen germination test conducted on media with different agar concentrations subjected to various time points of incubation</th>
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<tbody>
<tr>
<td>Agar Concentrations</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Concentration 12.5%</td>
</tr>
<tr>
<td>Concentration 6.25%</td>
</tr>
<tr>
<td>Concentration 2.5%</td>
</tr>
</tbody>
</table>

○ = Petri dish containing the pollens

After that, the petri dishes were observed under light microscope. Any visible pollen grains were counted and the percentage of in vitro germination were calculated. The pollen grains were considered germinated when then pollen tube length was greater than the diameter of the pollen grain (Tuinstra & Wedel, 2000). The percentage of germination was calculated as below: