INFLUENCE OF DIFFERENT EXTRACTION TEMPERATURES AND METHANOL CONCENTRATIONS ON FLAVONOID CONTENT OF *ACACIA AURICULIFORMIS* AND ITS ANTIFUNGAL AND ANTIOXIDANT ACTIVITIES

THAM MUN WAI

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2014

Declaration

I declare this dissertation my own work except for the quotations, excerpts, summaries and references which I had acknowledged the original source and author.

15 June 2014

Tham Mun Wai
PF2009-8081

CERTIFICATION

NAME	:	**THAM MUN WAI**
MATRIC NO.	:	**PF2009-8081**
TITLE	:	**INFLUENCE OF DIFFERENT EXTRACTION TEMPERATURES AND METHANOL CONCENTRATIONS ON FLAVONOID CONTENT OF *ACACIA AURICULIFORMIS* AND ITS ANTIFUNGAL AND ANTIOXIDANT ACTIVITIES**
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1. SUPERVISOR

Dr. Liew Kang Chiang Signature

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ABSTRACT

Acacia auriculiformis is a fast growing tree species widely grown in plantations. It contains an abundance of natural products including flavonoids possessing antioxidant, antifungal and other biological activities. These flavonoids can only be fully utilized after it is extracted optimally. With this in mind, the main objectives of this study were to determine the optimum extraction temperatures and methanol solvent concentrations on the *A. auriculiformis* heartwood (HW) and bark (B) flavonoids extraction yield and its antioxidant and antifungal activity. The wood samples were collected, air dried under shelter for 2 weeks, flaked, grinded and sieved to a pulverized form. These pulverized wood samples were extracted in a ratio of 1:20 [pulverized samples (g): methanol solvent (mL)] at 50 rpm for 3 hours in a water bath shaker with different extraction temperatures (35°C, 55°C and 75°C) and methanol solvent concentrations [55% (v/v), 65% (v/v) and 75% (v/v)]. The extraction yields [total extractives (TE), total phenolics (TP) and total flavonoids (TFlav)], antioxidant (DPPH free radical scavenging activity) and antifungal (mycelia growth inhibition) activities of these extracts were then evaluated. The antifungal activity of the extracts were done against the following wood rotting fungi; *Coniophora puteana* (CP), *Stereum ostrea* (SA), *Pycnoporus sanguineus* (PS), *Trametes spp* (M) and *Microporus xanthopus* (MX). The results showed that an increase in the extraction temperatures and methanol solvent concentrations increases the *A. auriculiformis* heartwood and bark extraction yields where the optimum extraction yields [HW (TE–9.81%, TP–75.44% and TFlav–36.64%) and B (TE–18.89%, TP–87.18% and TFlav–99.10%)] were achieved at an extraction temperature of 75°C with a 75% methanol solvent concentration. Statistical analysis using Pearson correlation coefficient also indicated that there is a positive correlation between the extraction temperatures and methanol solvent concentrations with the extraction yields. The extracts extracted at an extraction temperature of 75°C with a 75% methanol solvent concentration also exhibited the highest antioxidant [HW (IC_{50} value–2.23 mg/mL) and B (IC_{50} value–1.79 mg/mL)] and antifungal [HW (CP–31.40%, PS–12.80%, M–17.01%, MX–0.00% and SA–49.55%) and B (CP–41.60%, PS–21.27%, M–27.55%, MX–0.00% and SA–48.65%)] activity which increases with an increase in the extraction temperatures and methanol solvent concentrations. This increase in the antioxidant and antifungal activity were influenced by the increase in the extraction yields of the extracts tested which was further supported by the statistical analysis using Pearson coefficient correlation indicating that there is a positive correlation between the extraction yields with the antioxidant and antifungal activity exhibited by the extracts. These results show that *A. auriculiformis* heartwood and bark flavonoids extracts to be an invaluable source when incorporated into the development of an environmental friendly wood preservatives.

ABSTRAK

PENGARUH SUHU PENGEKSTRAKKAN DAN KEPEKATAN PELARUT METANOL DALAM PENGEKSTRAKKAN SEBATIAN FLAVONOID DARI ACACIA AURICULIFORMIS DAN APLIKASINYA SEBAGAI ANTIKULAT DAN ANTIOKSIDAN

Acacia auriculiformis adalah sejenis pokok malar hijau yang ditanam secara meluas di ladang. Pokok ini mengandungi pelbagai jenis sebatian kimia organik termasuk sebatian flavonoid yang mempunyai sifat-sifat biologi seperti antioksidan, antikulat dan sebagainya. Sebatian flavonoid ini hanya dapat dimanfaatkan setelah sebatian ini telah diekstrak secara optimum. Oleh itu, kajian ini telah dijalankan dengan objektif untuk mengenalpasti suhu pengekstrakkan dan kepekatan pelarut metanol yang optimum dalam pengekstrakkan sebatian flavonoid dari kayu teras (HW) dan kulit kayu (B) pokok A. auriculiformis dan aplikasinya sebagai antioksidan dan antikulat. Sampel kayu telah diambil, dikeringkan di bawah teduh selama 2 minggu untuk diproses menjadi serbuk kayu yang kemudiannya telah diekstrak dalam nisbah 1:20 [serbuk kayu (g): pelarut metanol (mL)] pada 50 rpm selama 3 jam dengan suhu pengekstrakkan (35°C, 55°C dan 75°C) dan kepekatan pelarut metanol [55% (v/v), 65% (v/v) dan 75% (v/v)] yang berbeza. Proses pengekstrakkan ini telah dijalankan di dalam sebuah water bath shaker. Kandungan sebatian kimia organik ekstrak [jumlah sebatian ektraktif (TE), fenolik (TP) dan flavonoid (TFlav)] telah ditentukan bersama dengan aplikasinya sebagai antioksidan (DPPH based free radical scavenging activity) dan antikulat (mycelia growth inhibition). Kulat pereput kayu yang telah digunakan dalam pengujian antikulat ekstrak adalah seperti yang berikut; Coniophora puteana (CP), Stereum ostrea (SA), Pycnoporus sanguineus (PS),Trametes spp (M) dan Microporus xanthopus (MX). Keputusan kajian ini menunjukkan bahawa peningkatan dalam suhu pengekstrakkan dan kepekatan pelarut methanol akan meningkatkan kandungan sebatian kimia organik ekstrak sampel kayu di mana pengekstrakkan sebatian kimia organik optimum [HW (TE–9.81%, TP–75.44% dan TFlav–36.64%) dan B (TE–18.89%, TP–87.18% dan TFlav–99.10%)] telah dicapai pada suhu pengekstrakkan 75°C dengan 75% kepekatan pelarut metanol. Analisis statistik menggunakan pekali korelasi Pearson juga menunjukkan terdapatnya hubungkait positif di antara suhu pengekstrakkan dan kepekatan pelarut methanol dengan kandungan sebatian kimia organik ekstrak. Ekstrak yang diekstrak pada suhu pengekstrakkan 75°C dengan 75% kepekatan pelarut methanol juga menunjukkan aktiviti antioksidan [HW (Nilai IC_{50}–2.23 mg/mL) dan B (Nilai IC_{50}–1.79 mg/mL)] dan antikulat [HW (CP–31.40%, PS–12.80%, M–17.01%, MX–0.00% dan SA–49.55%) dan B (CP–41.60%, PS–21.27%, M–27.55%, MX–0.00% dan SA–48.65%)] yang tertinggi di mana aktiviti antioksidan dan antikulat ekstrak meningkat seiring dengan peningkatan di dalam suhu pengekstrakkan dan kepekatan pelarut metanol. Peningkatan aktiviti antioksidan dan antikulat ini telah dipengaruhi oleh kandungan kimia sebatian organik ekstrak yang meningkat. Ini disokong oleh analisis statistik pekali korelasi Pearson yang menunjukkan terdapatnya hubungkait positif di antara kandungan kimia sebatian organik ekstrak dengan aktiviti antioksidan dan antikulat ekstrak. Daripada keputusan kajian ini, sebatian ekstrak flavonoid dari kayu teras dan kulit kayu pokok A. auriculiformis didapati sesuai untuk dijadikan pengawet kayu yang lebih mesra alam.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Acacia auriculiformis belongs to the family Leguminosae (Wagner *et al*., 1999), is an evergreen fast growing tree species capable of growing vigorously up to 30 m in height. This tree species is widely grown throughout Southeast Asia including Sabah in plantations as a raw material for the production of wood composite and pulp and paper due to its fast growing nature. It is also often planted as a shade and ornamental tree for its small and attractive bright yellow flowers (Gilman and Watson, 1993).

It is also rich of natural products including a wide array of flavonoids such as 3,4′,7,8-Tetrahydroxyflavanone, teracacidin (Mihara *et al*., 2005), auriculoside, Oritin-4β-ol and 4,2′,3′,4′-Tetrahydroxychalcone (Harborne and Baxter, 1999) where some of these flavonoids possess antioxidant properties (Barry *et al*., 2005) which enables it to scavenge free radicals produced by the fungi and its extracellular fungal enzymes resulting in the inhibition of the fungal growth (Mihara *et al*., 2005).

These low moleclular weight flavonoids which have a common chemical structure, benzo-ɣ-pyrone (Sathishkumar *et al*., 2008) also possesses other biological properties including anti-inflammatory (Garcia-Lafuente *et al*., 2009), anti-depressent (Nisar *et al*., 2010) and cancer preventive properties assisting in the development of medications for cancer treatment (Zhang *et al*., 2005) as well as the potential to replace sucrose (Kinghorn *et al*., 2010) and synthetic based antifeedants (Drijfhout and Morgan, 2010) as sweeteners and antifeedants of natural origin.

All these biological properties exhibited by these naturally occurring flavonoids had attracted an increasing interest in it (Gattuso *et al.*, 2007) with more than 13,000 articles and books published on flavonoids specifically on its chemistry, biochemistry, pharmaceutical and nutritional role since 1990. Bearing this in mind, this study was conducted to investigate and determine the optimum extraction yield from *A. auriculiformis* heartwood and bark in relation with the extraction temperatures and methanol solvent concentrations. These extracts were later tested for its antifungal activity against wood rotting fungi and its antioxidant activity.

1.2 Justification

Antifungal resistance is becoming an important and pressing issue in the wood based industry where there is an urgent need to enhance the durability of the wood products especially with the climatic and the biological conditions in tropical countries providing a conducive environment, a hot and humid weather to facilitate the decomposition of the wood products by wood rotting fungi (Eriksson *et al.*, 1990; Zabel and Morrell, 1992; Eaton and Hale, 1993; Blanchette *et al.*, 2004). Other than that, this urgent need for an effective and environmentally friendly wood preservative arises due to the limited number of highly durable tropical tree species and the ineffectiveness of the synthetic based wood preservatives against a wide variety species of wood rotting fungi as well as its hazardous nature towards the environment causing environmental problems (Yen and Chang, 2008).

The search for an effective and environmentally friendly wood preservative had led us to the chemical compounds found abundantly in plants. These naturally occurring chemical compounds were found to exhibit antifungal activities against several wood rotting fungi with such specificity, species or group without harming other beneficial living microorganisms along the target wood rotting fungi and its biodegradability making it ideal for its implementation in modern pest management (Barnes, 1992; Arango *et al.*, 2005). This was shown by the antifungal activity exhibited by these two naturally occurring flavonoids, 3,4',7,8-

Tetrahydroxyflavanone and teracacidin against the following wood rotting fungi, *Phellinus noxius* and *Phellinus badius* respectively (Barry *et al*., 2005).

However, these flavonoids can only be fully utilized after it is extracted optimally (Chen *et al*., 2012; Guo *et al*., 2013; Savic *et al*., 2013). This is where this study plays an important role by providing the necessary scientific basis for the optimum extraction temperatures and methanol solvent concentrations in the *A. auriculiformis* heartwood and bark flavonoids extraction. The extracts antioxidant (DPPH based free radical scavenging) and its antifungal (mycelia growth inhibition) activity against the following 5 wood rotting fungi, *Coniophora puteana*, *Stereum ostrea*, *Trametes spp*, *Microporus xanthopus* and *Pycnoporus sanguineus* were determined offering an alternative source in the development of an effective and environmentally friendly wood preservative.

1.3 Objectives

The objectives for this study were:

a) To determine the influence of extraction temperatures and methanol solvent concentrations on the *A. auriculiformis* heartwood and bark extraction yields.

b) To determine the correlation between the *A. auriculiformis* heartwood and bark extraction yields with its antifungal activity against wood rotting fungi by using Poisoned Food Technique.

c) To determine the correlation between the *A. auriculiformis* heartwood and bark extraction yields with its antioxidant activity (DPPH based free radical scavenging).

CHAPTER 2

LITERATURE REVIEW

2.1 Woody Plants

2.1.1 Trees

Trees including *A. auriculiformis* produce phytochemicals through its specialized metabolic function living cells which will then be deposited within the trees especially in its stem and bark to enhance its resistance against harmful microorganisms (Rowell *et al*., 2005). These phytochemicals distribution in trees differs by tree species and also by the tree components; sapwood, heartwood and bark (Figure 2.1) where most of it is abundantly found in the heartwood and bark (Gierlinger and Wimmer, 2004).

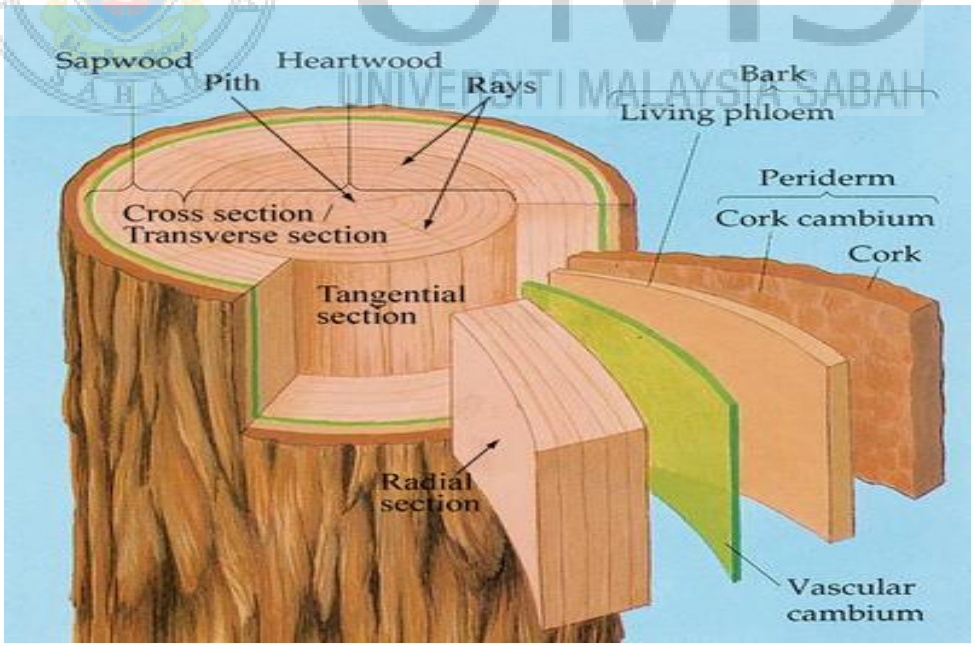

Figure 2.1 : A diagram of a tree stem including sapwood, heartwood and bark.

Source : Anon (2012)

The different distribution of phytochemicals is useful in taxonomic classification of trees (Evert, 2006) and it also imparts a darker colouring to the heartwood compared with the surrounding sapwood as is illustrated in Figure 2.2 (Patten *et al.*, 2010). More importantly, studies had also shown that these phytochemicals have a diverse range of usages such as sweeteners (Kinghorn and Soejarto, 1986; Nanayakkara *et al.*, 1988; Kinghorn *et al.*, 2010), antifeedants (Ohmura *et al.*, 2000; Morimoto *et al.*, 2006; Isman, 2006; Drijfhout and Morgan, 2010), food flavourings, fragrance chemicals and medications (Patten *et al.*, 2010).

Figure 2.2 : Cross-sections of three selected heartwood forming trees, (a) Western Red Cedar (*Thuja plicata*), (b) Ebony (*Diospyros spp.*) & (c) Lilac (*Syringa vulgaris*).

Source : Patten *et al.*, (2010)

2.1.2 *Acacia auriculiformis*

A. auriculiformis of Leguminosae plant family are also known as auri, earleaf acacia, Earpod wattle, Northern black wattle, Papuan wattle and Tan wattle (PIER, 2002). It is economically important for its timber (Pinyopusarerk *et al.*, 1991; Ishiguri *et al.*, 2004; Chowdhury *et al.*, 2005; Kabir and Webb, 2005) and is also used in the afforestation of wastelands (Islam *et al.*, 1999) due to its quick growing nature (Chowdhury *et al.*, 2013) with a high germinative capacity (Krishnan, 2010). Besides that, it also contains phytochemicals including phenolics and flavonoids (Harborne and Baxter, 1999) exhibiting strong biological activities such as antifungal and antioxidant activity (Barry *et al.*, 2005; Mihara *et al.*, 2005).

2.2 Extraction Method

2.2.1 Solvent Extraction

It is a commonly used method in extracting wanted/unwanted chemicals (solutes) from plant materials using a solvent (Cox and Rydberg, 2004). This method involves the plants samples (solid phase) immersed in a solvent (liquid phase) for a certain period of time (Houghton and Raman, 1998) to allow the solutes to distribute itself between these 2 phases (Figure 2.3). The solvent used should have a high solubility for the solutes enabling it to extract the solutes while co-extracting a minimum of impurities (Marcus, 2004). One such example is the usage of hexane or petroleum ether (solvent) to specifically extract fats or chlorophyll (solutes) from the seeds or leaves of the plant material respectively (Beek, 1999).

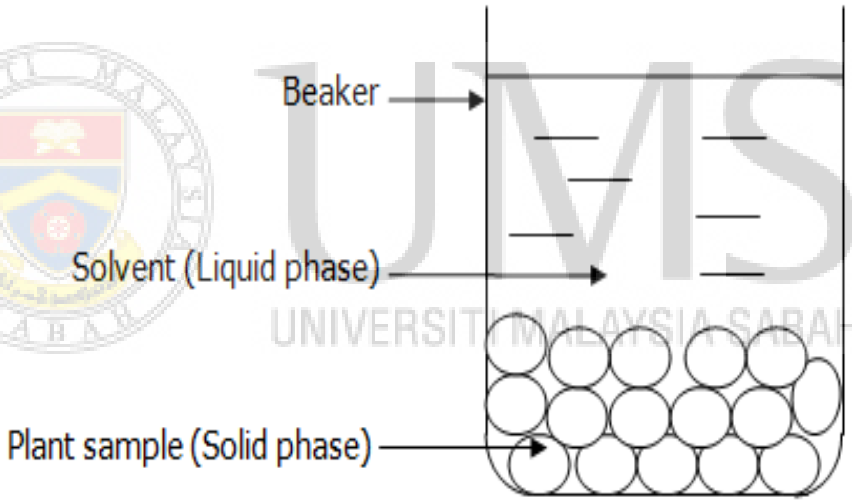

Figure 2.3 : Solvent Extraction (Solid-Liquid Phase).

Therefore, the selection of solvent is important in an extraction process (Sultana *et al.*, 2009; Jakopic *et al.*, 2009). Numerous studies had been done to determine the solvent that yields the highest phenolics and flavonoids from different plant materials (Table 2.1). Based on these reported findings, methanol solvent was chosen to extract phenolics and flavonoids (solutes) from *A. auriculiformis* heartwood and bark in this study.

Table 2.1 : Effect of solvents on the phenolics and flavonoids yields from extraction different plant materials

Reference	Plant Material	Solvents	Extraction Yields	
			Phenolics (Solutes)	Flavonoids (Solutes)
Stankovic, 2011	*Marrubium peregrinum* L. (Whole herb)	Methanol	49.27±0.815 mg GAE/g extract	54.77±0.598 mg RE/g extract
		Water	46.78±0.258 mg GAE/g extract	18.72±0.417 mg RE/g extract
		Ethyl acetate	33.51±0.616 mg GAE/g extract	51.33±0.793 mg RE/g extract
		Acetone	48.72±0.407 mg GAE/g extract	53.47±0.940 mg RE/g extract
		Petroleum ether	27.44±0.556 mg GAE/g extract	22.92±0.386 mg RE/g extract
Gautam *et al*., 2012	*Murraya paniculata* Linn. (Leaves)	Methanol	24.8±0.64 µg GAE	2.11±0.23 µg QE
		Ethanol	15.4±0.38 µg GAE	1.62±0.18 µg QE
		Petroleum ether	13.5±0.96 µg GAE	3.38±1.89 µg QE
		Hydro-alcoholic	9.06±1.13 µg GAE	1.80±0.21 µg QE
Borkataky *et al*., 2013	*Eclipta alba* (L.) Hassk. (Whole herb)	Ethanol	8.933±0.231 mg GAE/g extract	30.667±1.155 mg RE/g extract
		Ethyl acetate	4.267±0.231 mg GAE/g extract	27.333±1.155 mg RE/g extract
		Petroleum ether	2±0 mg GAE/g extract	8±0 mg RE/g extract
		Aqueous	1.467±0.231 mg GAE/g extract	0.5±0 mg RE/g extract
Ghasemzadeh *et al*., 2011	*Zingiber officinale* Roscoe Halia bentong variety (Leaves)	Methanol	33.1±1.21 mg GAE/g dry weight	5.5±0.54 mg QE/g dry weight
		Acetone	30.1±0.22 mg GAE/g dry weight	4.7±0.55 mg QE/g dry weight
		Chloroform	28.8±0.21 mg GAE/g dry weight	4.54±0.64 mg QE/g dry weight
	Zingiber officinale Roscoe Halia bentong variety (Stems)	Methanol	7.8±0.89 mg GAE/g dry weight	1.3±0.12 mg QE/g dry weight
		Acetone	7.2±1.05 mg GAE/g dry weight	0.83±0.14 mg QE/g dry weight
		Chloroform	7.07±0.99 mg GAE/g dry weight	0.74±0.102 mg QE/g dry weight
	Zingiber officinale Roscoe Halia bentong variety (Rhizomes)	Methanol	10.1±0.21 mg GAE/g dry weight	3.6±0.12 mg QE/g dry weight
		Acetone	9.8±0.22 mg GAE/g dry weight	3.4±0.13 mg QE/g dry weight
		Chloroform	9.2±0.66 mg GAE/g dry weight	3.23±0.12 mg QE/g dry weight
	Zingiber officinale Roscoe Halia Bara variety (Leaves)	Methanol	39.06±1.62 mg GAE/g dry weight	7.05±1.67 mg QE/g dry weight
		Acetone	34.6±1.88 mg GAE/g dry weight	6.2±1.71 mg QE/g dry weight
		Chloroform	33.6±1.99 mg GAE/g dry weight	6.01±1.65 mg QE/g dry weight
	Zingiber officinale Roscoe Halia Bara variety (Stems)	Methanol	8.5±1.02 mg GAE/g dry weight	1.7±0.49 mg QE/g dry weight
		Acetone	8.06±0.92 mg GAE/g dry weight	0.95±0.2 mg QE/g dry weight
		Chloroform	8.8±0.82 mg GAE/g dry weight	0.9±0.16 mg QE/g dry weight
	Zingiber officinale Roscoe Halia Bara variety (Rhizomes)	Methanol	13.4±0.34 mg GAE/g dry weight	4.4±0.57 mg QE/g dry weight
		Acetone	11.1±0.87 mg GAE/g dry weight	3.8±0.12 mg QE/g dry weight
		Chloroform	10.8±0.75 mg GAE/g dry weight	3.7±0.15 mg QE/g dry weight

Note :
RE – Rutin equivalent
QE – Quercetin equivalent
GAE – Gallic acid equivalent
Each value is the average of triplicate measurements±standard deviation

2.2.2 Influence of Extraction Parameters on Extraction Yields

Extraction parameters such as extraction time, material ratio, extraction temperature and solvent concentration affects the extraction yields independently or interactively (Wang *et al*., 2004; Kosar *et al*., 2005; Liyana-Pathirana and Shahidi, 2005). The 2 extraction parameter that has the greatest impact on the extraction

yields are extraction temperature and solvent concentration (Bucic-Kojic *et al.*, 2011). It is based on this that the extraction temperature and solvent concentration was chosen for evaluation in this study.

2.2.3 Influence of Extraction Time on Extraction Yields

Theoretically, the extraction yields will increase with the extraction time as this will allow sufficient time for the solvent to extract the chemicals (solutes) from the plant materials (Spigno *et al.*, 2007). This was observed in the work of Sathishkumar *et al.* (2008) where the flavonoids extraction yield increased from 110.6 mg/g tissue to 137.4 mg/g tissue as the extraction time was increased from 1 to 2 hours.

However, prolong extraction time may increase the loss of solvent by vaporisation (Dent *et al.*, 2013). This will create a more concentrated solvent which would eventually reduce the extraction yields (Uma *et al.*, 2010). Therefore it is suggested that an extraction time of no longer than 3 hours is employed (Xu *et al.*, 2005; Durling *et al.*, 2007).

2.2.4 Influence of Material Ratio on Extraction Yields

Material ratio (plant material, g: solvent, mL) has a positive effect on the extraction yields (Zhang *et al.*, 2007). A higher material ratio increases the concentration gradient between the plant material and the solvent which promotes the chemicals (solutes) rapid diffusion into the solvent (Kim *et al.*, 2004). Thus, increasing the extraction yields of the plant material (Cacace and Mazza, 2003; Al-Farsi and Lee, 2008).

This is supported by the findings of Tan *et al.* (2011) where the total phenolics and flavonoids yield from *Centella asiatica* increases with an increase in its material ratio from 1:05 to 1:20. Similarly, the material ratio of 1:20 was also shown to optimize the extraction yields of other plant materials (Xu *et al.*, 2005; Cai *et al.*, 2010; Radojkovic *et al.*, 2012). However, the extraction yields will cease to

increase once equilibrium is reached (Herodez *et al.*, 2003; Sathishkumar *et al.*, 2008). Therefore, a material ratio of 1:20 was selected for this study.

2.2.5 Influence of Solvent Concentration on Extraction Yields

Many studies showed that the extraction yields increases with the solvent concentration until a further increase in the solvent concentration decreases the extraction yields (Cacace and Mazza, 2003; Luthria and Mukhopadhyay, 2005; Durling *et al.*, 2007; Al-Farsi and Lee, 2008). These results can be explained by the 'like dissolve like' or 'polarity versus polarity' principle which states that solvents only extract chemicals that has the same polarity with it (Chew *et al.*, 2011). The addition of water to a solvent usually creates a more polar medium until the polarity of both the solvent and the chemicals (solutes) coincide (Spigno *et al.*, 2007).

This will facilitate in the extraction of the chemicals (solutes) from the plant material (Uma *et al.*, 2010). Thus, increasing the extraction yields as the solvent concentration was increased (Sathishkumar *et al.*, 2008). The presence of water in a solvent mixture also helps to swell the plant material, allowing an efficient transfer of the solvent into the plant material (Ong and Law, 2012). This increases the extraction yields too (Luthria and Mukhopadhyay, 2005). However, further increase in the solvent concentration decreases the extraction yields as both the solvent and the chemicals (solutes) are no longer of the same polarity (Sathishkumar *et al.*, 2008). Based on the literature review, the following solvent concentration, 55%, 65% and 75% was chosen to be evaluated in this study.

2.2.6 Influence of Extraction Temperature on Extraction Yields

An increase in the extraction temperature increases the extraction yields (Lim and Murtijaya, 2007; Silva *et al.*, 2007). It could be due to the greater speed of both the chemicals (solutes) (Xu *et al.*, 2005; Sathishkumar *et al.*, 2008) and the solvent molecule movements at higher extraction temperature (Houghton and Raman, 1998). This enables the chemicals (solutes) molecule to diffuse from the plant material into the solvent at a higher rate (Juntachote *et al.*, 2006) and at the same