ACCELERATED AQUEOUS EXTRACTION AND PHYTOCHEMICALS SCREENING OF *EURYCOMA LONGIFOLIA* (TONGKAT ALI) EXTRACT

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Dedicated to my beloved parents, brothers and my sisters, who had provided me with the support spiritually and emotionally throughout the long journey. To my dearest husband; Mat Salleh Yamin and my children; Muhammad Adam Haziq and Nur Aleysha Qurratu'aini, who had motivated me to complete the study.

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ABSTRACT

The development of a rapid, robust and reliable method for extraction of plant materials is important for the screening of a wide range of plant bioactives and the discovery of biomarker. Accelerated aqueous extraction or commercially known as Accelerated Solvent Extraction (ASE) is an automated extraction technique operated at elevated temperatures and pressures to achieve extraction in a short period of time. The high temperature weakens the solute-matrix interactions and leads to a faster diffusion rate, better analyte solubility and lower solvent viscosity. This research was undertaken to evaluate the performance of an accelerated aqueous extraction of eurycomanone and other bioactive compounds from Tongkat Ali. Investigation was carried out to elucidate the effect of static cycle, static time and temperature on the content and degradation of eurycomanone. To date, there is no study being carried out on optimization of the extraction of eurycomanone from Tongkat Ali roots using this technique. The optimum operating conditions were subsequently used for the extraction of other phytochemicals. Response surface methodology was used to determine the significant operating conditions. The Box-Behnken design was implemented to maximize the response (eurycomanone content) from the resulted response surface. The extraction yield of eurycomanone are mainly affected by temperature (>100 ^{o}C) followed by the static time. A higher static time (>11 min) was found to cause eurycomanone degradation, while a lower temperature and static time reduced the extraction efficiency. The optimum conditions yielded a corresponding eurycomanone content of 9.21mg/g at static time of 8 minutes, static cycle of 5 and temperature of 90 ^{o}C. A liquid chromatography coupled with a triple quadrapole and time-of-flight, mass spectrometer (LC-QTOF-MS/MS) was used to profile the small metabolites. The major quassinoid identified were 13α(21)-epoxyeurycomanone, eurycomanone, longilactone14, 15β-dihydroxyklaineanone, 6α-hydroxyeurycomalactone, eurycomalide B, laurycolactone A and laurycolactone B. In summary, the combination method of ASE and statistical analysis presented is an expedient technique for the phytochemicals screening of Tongkat Ali roots.

ABSTRAK

Pembangunan kaedah yang cepat, tahan lasak dan diyakini untuk mengekstrak tumbuhan adalah penting untuk menyaring pelbagai bioaktif dan penemuan penanda-bio sesuatu tumbuhan. *Accelerated Aqueous Extraction* atau secara komersial dikenali sebagai *Accelerated Solvent Extraction* (ASE) adalah teknik pengekstrakan automatik beroperasi pada suhu dan tekanan tinggi untuk mencapai pengekstrakan dalam tempoh yang singkat. Suhu yang tinggi melemahkan interaksi antara bahan larut-matriks dan menghasilkan kadar resapan yang cepat, analit melarut dengan lebih baik dan kelikatan pelarut yang rendah. Kajian ini dijalankan untuk menilai prestasi kaedah *accelerated aqueous extraction* untuk *eurycomanone* dan sebation bioaktif lain daripada Tongkat Ali. Kajian telah dijalankan bagi menjelaskan kesan pengaruh kitaran statik, masa statik dan suhu terhadap kandungan serta degradasi *eurycomanone*. Sehingga kini, tiada kajian dijalankan untuk mengoptimumkan pengestrakan *eurycomanone* dari Tongkat Ali menggunakan kaedah ini. Keadaan operasi yang optimum kemudiannya digunakan untuk mengekstrak fitokimia lain. Perisian *response surface methodology* (RSM) digunakan untuk menentukan keadaan operasi yang ketara. Reka bentuk *Box-Behnken* dipilih untuk memaksimumkan tindak balas (kandungan *eurycomanone*) dari lakaran *response surface* yang terhasil. Kandungan ekstrak *eurycomanone* lebih dipengaruhi oleh suhu (> 100^{o}C) diikuti oleh masa statik. Masa statik yang tinggi (> 11min) akan menyebabkan kandungan *eurycomanone* terdegradasi. Suhu serta masa statik yang lebih rendah akan mengurangkan kecekapan pengekstrakan. Keadaan optimum telah menghasilkan kandungan *eurycomanone* sebanyak 9.21mg/g pada masa statik 8 minit, 5 kitaran statik dan suhu 90 ^{o}C. Kaedah *liquid chromatography coupled with a triple quadrapole and time-of-flight tandem mass spectrometer* (LC-QTOF-MS/MS) digunakan untuk memprofilkan metabolit kecil. Kandungan *quassinoid* utama telah dikenal pasti iaitu *13α (21) -epoxyeurycomanone, eurycomanone, longilactone14, 15β-dihydroxyklaineanone, 6α-hydroxyeurycomalactone, eurycomalide B, laurycolactone A* dan *laurycolactone B.* Kesimpulannya, gabungan kaedah *ASE* dan analisis statistik yang dibentangkan merupakan teknik mudah untuk menyaring pelbagai fotokimia daripada akar Tongkat Ali.

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INTRODUCTION

1.1 General

Tongkat Ali, or *Eurycoma longifolia*, a traditional Malay and Orang Asli herb used as an aphrodisiac, general tonic, anti-Malarial, and anti-pyretic. Traditionally, the decoction of the Tongkat Ali roots is taken orally, for enhancing testosterone levels in men. It has also been used as herbal ingredient for women after child birth, for restoring energy and vitality and enhancing blood flow. Scientifically it has also been found to have anti-tumour and anti oxidant properties (Itokawa *et al.*, 1992; Jiwajinda *et al.*, 2002; Ang *et al.*, 1995(a) and (b); Kordono *et al.*, 1991; Kuo *et al.*, 2004; Chan *et al.*, 1986, 1989, 2005; Mohd Ridzuan *et al.*, 2005). Various chemical composition studies on Tongkat Ali have been carried out since the 1960's, mostly from the roots. The range of secondary metabolites found fall under the families of quassinoids, tirucallane-type triterpenes, squalene deriavatives, biphenynolignans, canthin-6-one and β-carboline alkaloids (Chan *et al.*, 1998; Kuo *et al.*, 2004; Jiwajinda *et al.*, 2002). The isolation of nearly sixty-five compounds from the roots of Tongkat Ali was reported by Kuo *et al.*, 2004 and Chua *et al.*, 2011.

1.2 Herbal Extraction

In herbal processing, extraction plays an important role as it is the first essential step for isolation and purification of many bioactive compounds. Herbal extraction processes are used to produce herbal extracts from the herbal raw material

in several forms that include liquid extracts which contain the soluble aspect of the plant material, oleoresins which contain the volatile and non-volatile plant components, and essential oils which only contain the volatile plant components. The common processes used for herbal extraction include batch solvent extraction, hydrodistillation and steam distillation, soxhlet, reflux, maceration, supercritical fluid extraction (SFE), ultrasonic assisted extraction (USAE), pressurised liquid extraction (PLE) and many others. Traditional extraction techniques are fairly simple, standard and continue have widespread use, but requires long extraction times and large amounts of samples, sorbents and organic solvents, of which the later are often costly (Camel, 2001; Mustafa and Turner, 2011). In addition, final extracts from the traditional extraction methods often require subsequent concentration and clean-up prior to analysis. Furthermore, the traditional extraction techniques would not be suitable when considering the extraction of bioactive compounds that are sensitive, thermolabile and found in low concentrations, due the fact that those compounds generally have selectivity with a probable low yields. The ideal extraction process is dependent on several factors. It should be quantitative, time saving and non-destructive. The choice of processing parameters are depends on the limits set by the compounds in order to avoid degradation of their functionality and bioactivity.

Traditionally, Tongkat Ali roots were boiled over a period of a few hours and the decoction was drunk when the volume of the water had reduced by a certain amount. Within the herbal industry, this process is carried out at a larger scale where the main extraction process is the extraction vessel or cooker. From a Chemical Engineering point of view, this process is a mass transfer process known as batch solid liquid extraction or leaching. Solid liquid extraction is defined as the use of a solvent to dissolve and remove a soluble fraction, known as the solute, from an insoluble, permeable solid (Gertenbach, 2001). On laboratory scale, Tongkat Ali up to now been mostly extracted by conventional methods: maceration (Chan *et al.*, 1998 and 2003; Asiah *et al.*, 2007; Chua *et al.*, 2011), reflux (Ismail *et al.*, 2005 and 2006; Kuo 2004) and solvent extraction (Chua *et al.*, 2011 and 2013; Ang *et al.*, 1998; Asiah *et al.*, 2007; Jiwajinda *et al.*, 2001). These techniques are time consuming and use of an environmentally unfriendly solvent, such as methanol. Therefore, the development of rapid, robust and reliable method for extracting plant materials is

important not only for discovery the biomarker but also for screening a wide range of plant bioactives.

Accelerated aqueous extraction or commercially known as Accelerated Solvent Extraction (ASE) or Pressurised Liquid Extraction (PLE) is an automated extraction technique using elevated temperatures and pressures to achieve extraction in very short periods of time. The high temperature leads to faster diffusion rate, better analyte solubility, lower solvent viscosities which weakening the solute-matrix interactions. Various controllable parameters like static cycle, static time and temperature are also advantages. The filtration of the final extract is being performed automatically during its collection. Compared to conventional extraction techniques, higher automation, extraction yields, recovery and a shorter duration can be achieved (Benthin *et al.,*1999; Jentzer, *et al.,* 2015). Recently, this technique has become more popular for extraction of nutraceuticals and other bioactive compounds, mainly due to the fact that it is automated with reduces extraction time and solvent consumption, and also requires minimal sample pre-treatment (Mustafa and Turner, 2011).

1.3 Phytochemicals Screening of Tongkat Ali

Phytochemicals refers as phytonutrients; chemicals or nutrients derived from plants; compounds found in plants that are not required for normal functioning of the body but that nonetheless have an active role in the amelioration of disease. Phytochemical profiling does not provide information about specific metabolites, rather consider a total profile/fingerprint as a unique pattern characterising a snapshot of metabolites in a particular cell/tissue. It is also define as a high-throughput, rapid, global analysis of samples to provide sample classification (Dunn *et al.,* 2005, Fiehn 2002). Phytochemicals can be classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's and many other. Secondary constituents are the remaining plant chemicals such as alkaloids (derived from amino acids), terpenes (a group of lipids) and phenolics (derived from carbohydrates) (Walton *et al.*, 1999).

Natural product extracts contain a wide variety of chemical compounds. Tongkat Ali itself has over 90 major chemical constituents identified (Malaysian Herbal Database, 2006) as listed in Appendix A. To assist in the quantitative as well as the qualitative determination of extract contents, various analytical techniques have been used. The precise identification of compounds in Tongkat Ali previously reported were using High-performance liquid chromatography (Chan *et al.,* 1998; Ang *et al.,* 2000), 1D and 2D NMR (Jiwajinda *et al.,* 2001; Ang *et al.,* 2002; Kuo *et al.,* 2003 and 2004; Bedir *et al.,* 2003) and Multichannel Artificial Lipid-Polymer Membrane sensor (Zhari *et al.,* 2005 and 2006). However, these methods usually require high concentration of compounds with high purity and also extensive analysis for identification. Presently, Chua *et al.,* 2011 reported the approach of LC-MS/MS based metabolites identification showed that the aqueous extract of Tongkat Ali has different profiles when extracted at different temperature and grown in different geographical environment.

1.4 Problem Statement

The uniformity of quality of the commercial preparations of Tongkat Ali is still questionable. As herbal medicine is complex system of mixtures, the plant source, conditions of growth and harvest time will undoubtedly affect the presence and concentration of the bioactive constituents, thus affecting the quality and efficacy of phytomedicine/ neutraceutical products (phytoproduct). Besides these factors, the extraction and drying methods used to process the herbal material and the type of solvent used for extraction will also affect the end composition of the finished products. As a result, commercially available extracts vary greatly in their quality and compositions and presently, there is no guarantee that a standardised processing strategy for the extracts will yield herbal medicine of consistent and acceptable quality (Schulz *et al.,* 1998). This variety poses analytical challenges, both for profiling multiple metabolites in parallel and for the quantitative analysis of selected metabolites. Consequently, the development of fast and effective analytical methods for phytochemicals fingerprinting of plant extracts is of high interest. In addition, the extraction process must ensure that the extract retains a phytochemical profile close to

that of the original plant matter. To have a complete idea of the bioactivity of extract, it become necessary to optimize the extraction process to achieve the broadest possible range of phytochemicals. Hence, a high degree of automated, fast, robust and reliable extraction process is of high interest.

In this study, given the importance to precise identification of compounds in Tongkat Ali, the extraction conditions and analyses of their bioactive compounds have been investigated. Due to the fact that the ideal extraction process is dependent on several factors, the development of extraction methods to increase the yield of the desired bioactive compounds from Tongkat Ali is very important. Therefore, this research was undertaken to evaluate an accelerated aqueous extraction of eurycomanone content and other bioactive compounds from Tongkat Ali and to investigate how static cycle, static time and temperature influence the content and degradation of eurycomanone. Beside, up to present, there was no study done on optimizing eurycomanone compounds from Tongkat Ali roots using accelerated aqueous extraction. The most optimum operating condition then further analysed for screening their phytochemicals by direct analysis of crude extract solutions without any preliminary chromatographic separation step.

1.5 Objective of the Study

This study was to optimize and validate of a method for extraction and direct analysis of the biomarker and other bioactive compounds. Response Surface Methodology (RSM) was used on the accelerated aqueous extraction of Tongkat Ali roots to understand the impact on eurycomanone yield of the main parameters (static cycle, static time and extraction temperature). An additional optimisation and experimental validation were then undertaken to set up the accelerated aqueous extraction method retained followed by the phytochemical analysis of the extract using liquid chromatography tandem mass spectrometry. The objective of the study can be drawn as listed below:

1. To evaluate an accelerated aqueous extraction of eurycomanone content and other bioactive compounds from Tongkat Ali and to investigate how static cycle, static time and temperature influence the content and degradation of eurycomanone yield.
2. To determine the most optimum operating conditions of accelerated aqueous extraction in order to maximise the eurycomanone yield from Tongkat Ali root using response surface methodology (RSM).
3. To identify bioactive compounds of Tongkat Ali using liquid chromatography tandem mass spectrometry at optimum condition drawn from objective 2.

To achieve the objective, two major research scopes were carried out:

1. Extraction and optimization process of eurycomanone from Tongkat Ali.
2. Phytochemicals screening of Tongkat Ali extract.

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