RECOVERY OF O-COUMARIC ACID FROM *Gliricidia sepium* LEAVES USING ULTRASONIC-ASSISTED EXTRACTION FOR BIOCONVERSION OF DICOUMAROL AS POTENTIAL RODENTICIDE ANTICOAGULANT

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Professor Luqman Chuah Abdullah, PhD
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Gliricidia sepium is briefly a leguminous tree known as ‘pokok bunga Jepun’ which widely grown in Malaysia for fencing and shade. They are often associated with various traditional uses and medicinal values such as anti-inflammatory, anti-bacterial and anti-oxidant but very little research has been done on extraction for rodenticides application using this local bark size-plant to naturally reduce numerous rodents’ related problems. This research aims to develop an alternative to the chemical rodenticides that mostly used in agricultural sector. The work focuses on the two main processes including recovery of *o*-coumaric acid from ultrasonic assisted extraction and bioconversion into dicoumarol as superior anticoagulant compound through aerobic fermentation. Different experiments were designed to investigate the effects of various operating parameters on quantitative and qualitative aspects of *G. sepium* leaf extract. The effect of drying temperature on the quality of *G. sepium* leaf and drying kinetics were evaluated for determination of the

optimum drying temperatures. The optimum drying temperatures of *G. sepium* leaves was 50°C as *o*-coumaric acid was chemically degraded and decrease in concentration above this temperature. Midilli model was the best suit model among the selected thin layer models in predicting the drying process. Based on the optimization analysis using RSM, it was found that 36.14 ±0.015 mg L^{-1} *o*-coumaric acid was produced with the optimum extraction temperature of 47°C, extraction time of 45 min and ratio of solvent to solid as of 10:1 (mL g^{-1}). The study of bioconversion of *o*-coumaric acid to dicoumarol was conducted using fungal strain of *Aspergillus fumigatus* Fresenius NRRL 163 through submerged culture system. The fermentation process was done using shake flasks under optimized conditions (defined media, initial carbon and nitrogen concentrations at 10 and 2 g L^{-1}) with constant parameters (temperature of 55°C and agitation 150rpm) which resulted in maximum dicoumarol and cell concentration of 0.322 U L^{-1} and 9.33 g L^{-1} respectively. Dicoumarol was chromatographically identified using HPLC with retention time of 2.29 minutes, characterized using FTIR and its molecular ions were confirmed using LCMS/MS as 336.6 m/z. The rodenticide anticoagulant activities were proven through in vitro anticoagulant assays using Activated Partial Thromboplastin Time (aPTT) and Prothrombin Time (PT) tests in which the dicoumarol fraction demonstrated its potential anticoagulant activity with its ability to lengthen the coagulation time in aPTT test 43.8% higher than crude extract. The bioconversion process converting *o*-coumaric acid from *G. sepium* leaf extract into dicoumarol bioactive compound was successfully achieved indicating the plant potential as a source of natural rodenticide anticoagulant.

Keywords: bioconversion, dicoumarol, *o*-coumaric acid, kinetics, anticoagulant.

SDG: GOAL 9: Industry, Innovation and Infrastructure

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGAMBILAN SEMULA ASID O-KUMARIK DARIPADA DAUN *Gliricidia sepium* MENERUSI TEKNIK PENGEKSTRAKAN BERBANTU ULTRASONIK BAGI PENUKARAN DIKOMAROL YANG BERPOTENSI SEBAGAI ANTIKOAGULAN RODENTISID

Oleh

SITI ZALINA BINTI YAACOB

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Gliricidia sepium ialah pokok kekacang yang dikenali sebagai ‘pokok bunga Jepun’ banyak ditanam sebagai pokok pemagar dan peneduh. *G. sepium* sering dikaitkan dengan pelbagai kegunaan tradisional dan nilai perubatan seperti anti radang, anti bakteria dan anti oksida namun terdapat kekurangan penyelidikan yang dijalankan mengenai aktiviti antikoagulan rodentisid yang boleh digunakan untuk mengurangkan pelbagai masalah berkaitan tikus. Penyelidikan ini memberi tumpuan kepada dua proses utama iaitu pemulihan asid *o*-kumarik melalui pengekstrakan berbantu ultrasonik dan biokonversi dicoumarol sebagai sebatian antikoagulan yang unggul melalui penapaian aerobik. Eksperimen yang berbeza telah direka bentuk untuk menyiasat kesan pelbagai parameter operasi ke atas aspek kuantitatif dan kualitatif ekstrak daun *G. sepium.* Kesan suhu pengeringan terhadap kualiti daun *G. sepium* dan kinetik pengeringan telah dinilai untuk menentukan suhu pengeringan yang optimum. Suhu pengeringan optimum daun *G. sepium* ialah pada 50°C

kerana asid *o*-kumarik boleh terdegradasi secara kimia dan berkurang kepekatan jika melebihi suhu ini. Model Midilli merupakan model terbaik dalam kalangan model lapisan nipis terpilih dalam meramalkan proses pengeringan. Berdasarkan analisis pengoptimuman menggunakan RSM, didapati 36.14 ±0.015 mgL^{-1} asid o-kumarik dihasilkan dengan suhu pengekstrakan optimum 47°C, masa pengekstrakan selama 45 minit dan nisbah pelarut kepada pepejal pada 10:1 (mLg^{-1}). Kajian biokonversi asid *o*-kumarik kepada dikomarol telah dijalankan menggunakan strain kulat *Aspergillus fumigatus Fresenius* NRRL 163 melalui sistem kultur tenggelam. Proses penapaian dijalankan menggunakan medium tertakrif menggunakan kelalang goncang di bawah keadaan optimum (kepekatan karbon dan nitrogen awal pada 10 dan 2 gL^{-1}) di bawah parameter malar (suhu 55°C dan agitasi 150rpm) di mana nilai maksimum dikomarol and kepekatan sel masing-masing 0.322 UL^{-1} and 9.33 gL^{-1}. Dikomarol dikenal pasti secara kromatografi menggunakan HPLC dengan masa pengekalan 2.29 minit, dicirikan menggunakan FTIR dan ion molekulnya disahkan sebagi 336.6 m/z dengan menggunakan LCMS/MS. Aktiviti antikoagulan rodentisid telah dibuktikan melalui ujian antikoagulan in vitro menggunakan ujian Masa Tromboplastin Separa Teraktif (aPTT) dan Masa Protrombin (PT) di mana pecahan dikomarol menunjukkan potensi aktiviti antikoagulannya dengan keupayaannya untuk memanjangkan masa pembekuan dalam ujian aPTT 43.8% lebih tinggi daripada ekstrak mentah, Proses biokonversi asid *o*- kumarik dari ekstrak daun *G. sepium* kepada dikomarol telah dicapai dan berjaya membuktikan potensi tumbuhan ini sebagai antikoagulan rodentisid semulajadi.

Kata Kunci: antikoagulasi, asid *o*- kumarik, biokonversi, dikomarol

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LIST OF ABBREVIATIONS

DR	Drying rate (g/min)
M_e	Moisture content of the sample (%)
M_t	Moisture content at time t (%)
$M_t+\Delta t$	Moisture content of the previous time
MR	Moisture ratio
MR_{exp}	Observed moisture ratio
MR_{pre}	Predicted moisture ratio
R^2	Coefficient of correlation
RMSE	Root mean square error
w_i	Sample's initial weight (g)
w_f	Final weight (g)
k	Extraction rate constant (L/gmin)
E_a	Activation energy
A	Arrhenius constant
R_{SS}	Ratio solvent to solid (ml/g)
SSF	Solid State Fermentation
UAE	Ultrasonic Assisted Extraction
RSM	Response Surface Methodology
b_o	Regression coefficient
b_1	Linear regression coefficient
b_2	Linear regression coefficient
b_{11}	Squared regression coefficient
b_{12}	Interaction regression coefficient

CHAPTER 1

INTRODUCTION

1.1 A Worldview of Rodent Issues and Challenges

Rodents are a key mammalian group that is prominently dominant in most environments over the world. They are considered to be one of the most successful mammals due to their close association with humans which they are currently thought to be outnumber. In many instances, rodents offer major benefits for the environment as bio-engineers although according to the International Union for Conservation of Nature Red List Assessment 2017 by taxonomic order, the species is at risk, threatened or endangered. Among rodent groups, rats and mice are questionably the most essential family which hasplayed a central role in the human life cycle.

In many Third World nations, the rodent population is upward of 3 to 5%. As consumers of vitamins and protein-rich foods, rodents often feed on the crop embryos, stripping awaynutrients and germination capabilities. The creatures are notorious for contaminating foodsupplies and grain is one of their primary targets. In general, rats consume roughly 25 grams of paddy or rice grain on a daily basis, while mice eat approximately 3 to 4 g each day (IRRI Rice Knowledge Bank, 2017). While devouring these human food supplies, rats dispose of urine, faecal and pathogenic deposits that render the food inedible for humans.With their ever-growing teeth, rodents are constantly gnawing on things and are able to chew away at electricity power lines. Using their sharp incisors, the rodents gnaw at electrical wires and insulation materials, causing

short circuits and sparking fires.

Few decades ago, rodents have also been reported to be a major pest in the rice fields inSoutheast Asia (Singleton and Petch, 1994), Africa (Leirs *et al.,* 1997), Australia (Singletonand Redhead, 1989; Caughley *et al.*, 1998), and in many other parts of the world (Prakash,1988). In most Asian countries, rodents generally cause chronic losses to production in the order of 5 to 10 % per annum. In many rice plantation areas, this figure has risen dramatically over the past few decades, most noticeably in places where croppingfrequency has increased from one to two or three crops per year. It is common for smallholder rice farmers to report chronic yield losses of 20 to 30 % per annum, rising to 50 % or even total crop loss in certain seasons (Grant Singleton, 2003).

The ecology and management of rodent pests are rarely taught in universities and poorlyexplored generally. Expertise in rodent management is therefore lacking in most developing countries in Asia. Until a recent resurgence in interest and funding, rodent pestmanagement has not progressed in Asia since the 1970s mainly because there has been too little research effort to understand the biology, behaviour and habitat use of the species. However, there is a growing demand, particularly in developing countries, for rodent control strategies that either have less reliance on chemical rodenticides or have abetter target for their use.

1.2 Rodent Issues and Management Practices in Malaysia

Rodents are gnawing animals of the order rodentia, represented by four families in Peninsular Malaysia; *Muridae* (mice and rats), *Rhizomidae* (bamboo rats), *Sciuridae* (tree and ground squirrels) and *Hystricidae* (porcupines). Among the four families of rodents, rats and mice of the family *Muridae* have the highest species diversity, with 27 species sofar described in Peninsular Malaysia (Liat, 2015).

In Malaysia, all the four rodent species play a role as reservoir hosts for human diseases. Some of the common diseases encountered are *leptospirosis* (spirochaetal disease), *scrub* and *murine typhus* (ricketsial diseases), *angiostrongyliasis, schistomiasis, hymenolepsiasis, echinostomiasis* and *trichninellosis* (helminthic diseases), haemorrhagicfever with renal syndrome and *rat-bite fever* (Liat, 2015).

According to the annual report of Ministry of Health Malaysia, The Disease Control Divisionfrom 2014-2022, the increase in rodent population in Malaysia has attributed to high casesof *Leptospirosis* with high number of death from 5,370 diagnosed cases in 2015 due to infected urine of rodents and other domesticated animals. The ministry stated that it was in epidemic range since the incidence and case fatality of leptospirosis in Malaysia between 2014 and 2022 were 8.63–17.2 and 0.6–2.4% out of 100,000 populations, respectively. The states of Perak, Kelantan and Selangor alone contributed to the highest number of *Leptospirosis* cases, with Selangor registering 1,832 in 2014. Lack of concerted education for the public and prevention measures by the

Malaysian authorities against theimplosion of the rat menace perhaps best explains why a disease that caused only 20 deaths from 263 reported cases in 2004 has blown into 92 deaths from 7,806 diagnosed cases in 2020.

In Malaysia, a broad scale of chemical control is currently the primary method for rodent management. This control method has not, however, been widely adopted since farmers are used to free government distribution of acute poisons to make up their own bait, and they like to see dead rats, more likely to be left exposed with the poisons. Financial constraints are such that they cannot or will not spend far in advance of return. Furthermore, the use of chemicals in plantation areas such as palm oil or rice paddy fieldraises a number of concerns such as the risk to non-target species, the human health impact, the attack on the environment and the low efficacy of action when high food qualityis available.

1.3 Rodenticide Anticoagulants

Rodenticide anticoagulants were first discovered in the 1940s and have since become themost widely used toxicants for commensal rodent control (Hadler *et al.,* 1996). Rodents poisoned with anticoagulants die from internal bleeding as the result of a loss of blood clotting ability and damage to the capillaries. Prior to death, the animal exhibits increasing weakness due to blood loss, though appetite and body weight are not specifically affected.

The first anticoagulants (warfarin, pindone, diphacinone and clorophacinone), are commonly known as the first-generation anticoagulants or multiple-feed

rodenticides. Their enduring action requires multiple feedings over several days to a week to cause death. In order to achieve this multiple feeding, the bait must be made available on a continuous basis until the desired control is reached.

Warfarin resistance led to the development of the second-generation anticoagulants of dicoumarol. These compounds are much more potent than the first-generation anticoagulants, making them effective for the control of warfarin-resistant rats and mice. Animals that eat these rodenticides die from internal hemorrhaging (bleeding) within a few days. While all anticoagulants work in a similar way, second- generation products are more toxic and persistent in the bodies of rodents than first- generation ones and pose a greater threat to non-target wildlife and pets. As one feeding can produce death if a sufficient amount of bait is consumed, they are often referred to as single-feed anticoagulants. The effects of these compounds are also cumulative and will result in death after several feedings of even small amounts. As in the case of all anticoagulants, death is delayed for several days following the ingestion of a lethal dose.

Dicoumarol is a potent anticoagulant compound formed from coumarin by bacterial action in spoiled *Melilotus* species (Larry, 2015). Its discovery led to the development of modern anticoagulant drugs. Dicoumarol and warfarin, structurally related 2,3-epoxide reductase inhibitor, are the active ingredients in rat poisons and both hydroxylated in four positions (Kerry and Simon, 2013). This is deemed an essential requirement for powerful anticoagulant activity.

All of the common plant coumarins are not substituted at this position and therefore lack significant clinical anticoagulant activity, although many do possess very limited activity when consumed by animals in high dosages.

Dicoumarol can be found in spoiled sweet clover**,** which contains a mould that produces this metabolite and can cause fatal haemorrhagic episodes in animals that graze on hay containing this compound (Bryan, 2022). This coumarin derivative is used clinically as an anticoagulant to prevent thromboses case in patients prone to clot formation. Rodents thatconsume rodenticides containing dicoumarol anticoagulants generally require vitamin K treatment. The duration of antagonism depends on the amount consumed, as well as on the physiologic half-life of these compounds which can extend from days to weeks.

1.4 Problem Statements

Rodents are also one of the major causes of damage to agricultural crops throughout theworld. There are over 2,255 rodent species and 42 % of all mammalian species are classified as rodents. In Asia, a loss of 5 % of rice production amounts to approximately 30 million tonnes which would be able to feed 180 million people for 12 months (Grant Singleton, 2003). In Asia, rodents, insects and weeds are associated with causing similarlevels of pre-harvest and post-harvest losses of rice. The author also added that in some countries, such as Indonesia, rodents are economically the most important pre-harvest pest. In other countries like Vietnam, rodents are an emerging problem and are classifiedby farmers in the topmost three factors that shrink

the crop production while in most Asiancountries, wild rat has been known as a creature which destroys various agricultural products.

The common issue arises when chemical rodenticides are widely used to kill targeted rodents' population. These toxic materials are released intentionally into our environment which has led to severe impacts on the ecosystem. Various studies have proven that chemical rodenticides linger in the atmosphere, the ground and in the water system for a long period of time. Chemicals have been used on fields across the world for over a hundred years causing a build-up of adverse pollution in our (Smith and Shore, 2015).

Chemical rodenticides are also toxic to other living organisms. They can damage agricultural land by harming beneficial insect species, soil microorganisms and worms which naturally limit pest populations and maintain soil health. Chemical rodenticides weaken plant root systems and immune systems and reduce concentrations of essential plant nutrients in the soil such as nitrogen and phosphorous. Thus, in order to reduce suchproblems that are caused by chemical rodenticides, it is vital to find an approach that is environmentally friendly to develop biological rodenticides with anticoagulant properties for rodent control.

Another related problem is when rodents are also recognised as reservoirs of a wide spectrum of disease. They are identified to be disease carriers of which most are transmittable to humans, pets and other farm animals. This includes diseases such as *Leptospirosis, Eosinophilic, Meningitis, Murine Typhus*

Salmonella, tapeworms *Tuberculosis* and many more (Shapiro, 2017). The studiy indicates that rodents are the major carrier of more than 60 types of diseases such as Zoonosis that are transmissible to humans, animals and livestock. Rat bites and scratches for instance can result in rat- bite fever and other complications. The complications include renal and liver damage as well as cardiovascular problems. *Lymphocytic choriomeningitis*, a viral infectious disease,is transmitted through the saliva and urine of rats. The infected person might experience long-term effects or permanent discomfort.

The research on the natural anticoagulants is rarely explored for the rodenticides application and mostly focused to the herb species including *Zanthoxylum piperitum* (Hansen *et al.*, 2016), *Allium sativum* (Fischer *et al.,* 2013) and Capsicum species (Stefanini *et al.,* 2020). However, there are still lack of research been carried out on the local, bark-size, unlimited source of explants and non-seasonal species like *G. sepium*. Previous research had been done using the fifteen medicinal plants in Iran but mostly related to in vivo research (Alikhani, 2017) and the research were limited to the primary extracted compound without any bioconversion been carried out.

Therefore, the idea behind this research to explore the potential use of *Gliricidia sepium* as a rodenticide anticoagulant from natural source as an alternative to the chemical rodenticide anticoagulants are significant to be brought forward due to its environmentallyfriendly and low production cost. Bioconversion was applied to the primary extracted compound to enhance its anticoagulant properties. The enhanced properties of rodenticide

anticoagulants will then can serve as a green product for rodent control with specific bait formulation as an alternative to the commercialized chemical rodenticides.

1.5 Research Background

It is well known that most plants provide a wide range of the complex mixture of bioactivecompounds such as lipids, phytochemical, pharmaceutics, flavors, fragrances and pigments. Adverse usage of plant extracts had gained a lot of interest in the food, pharmaceutical, agriculture and cosmetics industries. Drying and extraction are the first key steps to obtain such valuable bioactive compounds from plants for further commercialization.

The processing of plant materials requires further investigation since itis the crucial step to have proper drying and extraction mechanism in order to ensure thetargeted plant could exhibit the optimum amount of desired compound. The extraction techniques can be divided into two groups, which are conventional and modern extraction techniques. Selection of an appropriate extraction technique lies on the amount of recovery, costs and efficiency of the process.

The interest in the investigation of bioactive compounds, especially phenolic compounds from plants has greatly increased in recent years. Phenolic compounds are considered assecondary metabolites that are synthesized by plants (Cosme, 2020). Simple phenols, phenolic acids, coumarins, flavonoids, tannins, lignans and lignins are included as phenolic compounds.

Gliricidia sepium is well known in plantation agriculture as a shade tree, causing it to be called the mother of cocoa. The plants were initially introduced into Sri Lanka from the West Indies and later to India (Rojas, 2022). In Malaysia, it is known as 'Pokok Bunga Jepun' and well recognized due to its beautiful flowers. The older generation believed thatthe powdered leaves, seeds and bark are poisonous when mixed with fermented rice or maize. It has been used as poison to kill pests such as rats and mice. Other than that, thejuices from the leaves can be used to reduce the pain due to centipede bite.

The toxic effects of *G. sepium* are well known for its native range in Central America, wherethe leaves or the ground bark, mixed with cooked maize, are used traditionally as a naturalrodenticide (Rojas, 2022). This toxicity is thought to be due to the microbial conversion of coumarin to dicoumarol during fermentation. In 1948, *o*-coumaric acid was isolated from the leaves ofsweet clover as a bioactive compound which was effective in killing rodents.

The study on bioconversion process is made to determine how *G. sepium* exerts its rodenticide properties. The extraction of the explants followed by physical and chemical fractionation reveals the presence of *o*-coumaric acid as a constituent of the phenolic fraction. Under the specified fermentation conditions, the bioconversion of *o*-coumaric acid by known fungal elementsinto the hemorrhagic agent of dicoumarol and in vitro anticoagulant assay gives an indication that *o*-coumaric acid can act as the basis for rodenticide properties of *G. sepium.*

The bioengineering approach is applied through the utilization of leaf extract of *G. sepium* containing *o*-coumaric acid as possible precursor for bioconversion to dicoumarol compound. The extract has been tested on the rat blood to reduce the blood ability to coagulate by lengthening the bleeding and coagulation time, reducing the number of thrombocytes and the amount of hemoglobin in the blood to further reduce rat population in the target area.

Technically, dicoumarol is a plant-derived compound which is chemically very similar to vitamin which interferes with the normal role of it in permitting the blood to clot. It might not be a fast-acting substance, but repeated doses with optimised conditions of plant extraction and fungal conversion will lead to fatal haemorrhages in rats within a few days. The right formulation of bioconversion process is expected to be able to enhance the rodenticide anticoagulant properties

1.6 Research Objectives

In a process of exploring the rodenticide anticoagulant activity of *G. sepium* leaf extract, this study embarks on the following objectives:

i. To investigate the effect of drying temperatures on the yield of *o*-coumaric acid and drying kinetic studies.

ii. To optimize the extraction of *o*-coumaric acid compound through UAE method using Response Surface Methodology (RSM) and its extraction kinetic.

iii. To optimize bioconversion process of dicoumarol from crude extract containing *o*-coumaric acid using *A. fumigatus* NRRL 63 through one factor at one-time method.

iv. To characterize extracts of *G. sepium* leaves and subsequent *Aspergillus fumigatus* Fresenius NRRL 63 bioconversion extracts respectively for in vitroanticoagulant activity.

1.7 Research Scope and Limitation

The scope of this work covers three key processes involved in producing a rodenticide anticoagulant compound initiated from *G. sepium* leaves extract which consist of raw material drying process, ultrasonic-assisted extraction extraction (UAE) and bioconversion of dicoumarol from *o*-coumaric acid bioactive compound. Drying of raw material is a principal step of all process which has a great influence on the extract quality. The effects of drying temperature on the quality of *G. sepium* and drying kinetics were studied in order to determine the optimum drying temperature and drying time. Thin layer models were used in describing the drying process. The effectiveness of UAE is affected by a number of operating parameters including type of solvent, extraction temperature, ratio of solvent to solid and time. The influence of solvent type on the quality and quantity of extract was evaluated by studying the extraction yield. The results were used for selection of the most suitable solvent. The effect of extraction temperature on the quality of *G. sepium* leaf extract was also studied.

The effects of the extraction temperature and ratio of solvent to solid were evaluated by comparing the extraction yields. The qualitative and quantitative results were used as guiding information for choosing a feasible extraction temperature and ratio of solvent to solid. The suitable duration for extraction

was determined by studying the extraction kinetics. Mathematical models were proposed as a way of predicting the extraction process.

The bioconversion process involves a fermentation step using *A. fumigatus* NRRL 163 under a submerge culture system followed by characterization of the converted dicoumarol compound. The fermentation process was conducted in a shake flask system at an optimum incubation time in producing the desired compound. The growth kinetics was investigated to relate the specific growth rate of the fungal population and the substrate concentration. The medium was finally purified through fractionation using HPLC and the compound was further characterized using FTIR and LCMS/MS.

1.8 Research Significance

The new approach of using a leguminous and non-seasonal local tree as an anticoagulant agent is an innovation to fully utilize the natural plants that are easily available, cheap and harmless to the environment. In agricultural sector, this work would benefit the agricultural practitioner in dealing with rice plantations especially by providing an alternative to the traditional rat control which is well recognized as the source of huge economic loss in the field.

This research will also reduce dependence on the chemical rodenticide anticoagulants for rat control which is currently widely applied by most agricultural practitioners. The use of natural rodenticides will minimize the issue of environmental contamination and other health attacks on animal species and human beings due to the severe effects of synthetic rodenticides.

The research findings will contribute in eliminating the zoonotic diseases carried by the rodent species causing health attacks within the community and farmers. Biological rodenticide anticoagulant from plant sources would have better safety margin andmay present with little side effects on other crops in agricultural areas. A better understanding of plant processes and rodent's ecology will provide a strong basis for cost effective and sustainable strategies for managing rodent pests. Thus, there is a primary need to investigate local medicinal plants in a view harnessing their anticoagulant properties for further use in agricultural practices

CHAPTER 2

LITERATURE REVIEW

2.1 *Gliricidia sepium*

Gliricidia sepium is a leguminous tree which belongs to the family *Fabaceae.* Its scientificname is *Gliricidia sepium* (Jacq.) Walp, which literally translates to “rat poison”. It originated in Central America and has spread to numerous parts of the world particularly South Asia. The plant is utilized for fuel wood, animal feed, green compost, shade, greenfences and as support plants. According to World Agroforesty, the leaves of *G. sepium* have a high feeding value with crude protein comprising 20 to 30 % of the dry matter, a crude fiber content of about 15 % and an in vitro dry matter digestibility of 60 to 65 %.

G. sepium was originated from Mexico and being introduced to Sri Lanka, mostly used inmany tropical and sub-tropical countries as live fencing plants. *G. sepium* is a small to medium sized tree which usually attains a height of 10-12m with composite leaves (Nuwarapaksha *et al.*, 2023). The branching of plant is usually from the base with a basaldiameter ranging from 50-70 cm and the fruit is a pod at about 10-15 cm of length. The bark of the plant is smooth and variable in colour from greyish to dark red brown. The flowers are reddish in colour and there are no leaves at the end of branches. Typically, it can be found growing in acidic soils with low to medium fertility.

The scientific name of *Gliricidia sepium* is derived from Latin *glis* (rat killer) and Spanish name of *mata-raton* refers to its rodenticidal properties *G. sepium* is

well known with Spanish name as *madre de cacao* (mother of cacao), refers to the plant's frequent use as cacao shade tree. In Malaysia, it is locally known as bunga Jepun as it is widely used for fencing and also as shade plants for cocoa and coffee. Its purplish pink colour of flowers can be seen as in Figure 2.1.

Figure 2.1 : *Gliricidia sepium* bark, leaves and flowers

2.1.1 Botanical Classification

Gliricidia sepium is characterized under below botanical classification.

Plant name : *Gliricidia sepium*

Kingdom : Planta

Order : Fables

Family : Leguminosae

Genus : *G. sepium*

Species : *G. sepiu*

2.1.2 Traditional Uses and Medicinal Values

Traditionally, *G. sepium* leaves can be used as a nutritional supplement for sheep and cattle as it contains 20-30% crude protein (Amata, 2014). *G. sepium* is planted as soil stabilizer in most contour hedgrows on sloping lands susceptible to soil erosion (Atapattu *et al.*, 2017). The hedgrows would hold soil structure and can slow erosive surface run- offif properly managed. *G. sepium* has been also used as intercropping for shade and organicmatter with cacao, coffee, vanilla, tea and other crops. (Nuwarapaksha *et al.*, 2023).

In medicinal perspective, *G. sepium* had been reported to be expectorant, insecticidal, rodenticidal, sedative, suppurative and folk remedy for alopecia, boils, bruises, burns, colds, cough, debility, eruptions, erysipelas, fever, fractures, gangrene, headache, itch, prickly heat, rheumatism, skin, sore, tumors, wounds, ulcers and urticaria (Orwa *et al.*, 2019). In Mexico, the plant is used as anthistaminic, antipyretic, expectorant and diuretic and the extracts have shown the high antibacterial activity (Stewart, 2016).

2.1.3 Bioactive Compounds and Bioactivities

There are many compounds have been identified and quantified from leaves of *G. sepium*by Gas Chromatography-Mass Spectrum analysis. They are found to be propylene glycol, coumarin, (Z)-3-hexenol, B-farnesene and (E)-2-hexenol (Kaniampady *et al.,* 2007).Fifteen allelochemicals from *G. sepium* had also been extracted, identified and quantified1993). They were identified as gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, *B*-resorcyclic acid, vanillic acid, syringic acid, *p*-coumaric acid, *m*-coumaric acid,

o-coumaric acid, ferulic acid, sinapinic acid (*trans* and *cis* forms), coumarin and myricetin. There are several reports on biological activities of *G. sepium* as summarized in Table 2.1.

Table 2.1 : Various solid liquid techniques using of *G. sepium* explants

***G. sepium* Explants**	**SLE Techniques**	**Activities and Application**	**References**
Leaves	Organic solvent extraction	Antibacterial	Juanico *et al.* (2023)
Leaves and stem	Organic solvent extraction	Natural antioxidant agent	Adekemi *et al.* (2021)
Leaves	Microwave assisted extraction	Pesticides against black bean aphids	Rhonalyn *et al.* (2021)
Flowers, bark, seeds and leaves	Soxhlet extraction	Phytochemicals	Dubal *et al.* (2020)
Leaves	Solvent extraction	Antibacterial in treatment of poultry pathogens.	Oladunmoye *et al.* (2018)
Leaves		Inhibitory activity in the growth of lettuce (*Lactuca sativa*) radicles.	Takemura *et al.,* (2012)
Leaves	Soxhlet extraction	Antibacterial against local bacterial species	Neethu *et al.,* (2016)
Leaves	Soxhlet extraction	Antibacterial & antioxidantagainst 9 clinical bacterial isolates	Akharaiyi *et al.* (2012)
Leaves	Maceration	Insecticidal against papaya mealybugs	Nismah *et al.* (2019)

It was believed that alleopathic plants or products derived from these allelochemicals will be less harmful to the environment compared with synthetic rodenticides because the former readily undergo degradation in the environment. Griffifths (1962) had reported through analytical values indicating that in *G. sepium* young leaves contains almost equalamounts of coumarin and *o*-coumaric acid whereas in the herbaceous plant, *Melilotus officinalis*, the ratio of coumarin to *o*-coumaric acid was 50:1. In relation to this, the

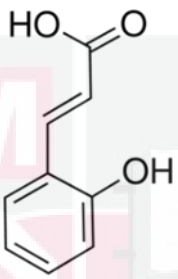
anticoagulant activity in *G. sepium* leaves is produced by using o-coumaric acid as bioactive compound for further use in development of an effective rodenticide.

2.1.4 *O*-Coumaric Acid

O-coumaric acid is a hydroxycinnamic acid, an organic compound that isa hydroxyl derivative of cinnamic acid. There are three isomers of coumaric acids namely *o*-coumaric, *m*-coumaric acid and *p*-coumaric acid. They differ by the position of the hydroxy substitution of the phenyl group. *O*-coumaric acid and other phenolic related compounds like melilotic acids have a metabolic role in the synthesis of dicoumarol and *o*-coumaric acid lies under group of phenolic acids that are widespread in the plant kingdom with biological activities, such as antioxidant, anti-inflammatory, antimicrobial, anticancer and anti-anxiety activities and have been widely used in the food, medicine and cosmetic industries. *O*-coumaric could also be found in many medicinal herbs including *Boehmeria nivea (L.) Gaudich*, *Salvia miltiorrhiza Bge., Ginkgo biloba L., Acanthopanax senticosus, Harms, Myristica fragrans Houtt* and *Cimicifuga foetida L.* (Xu *et al.,* 2017).

Table 2.2 shows the properties of *o-coumaric acid*.

Table 2.2 : Properties of *O*-Coumaric Acid

Properties	Descriptions
Other names	2-Hydroxycinnamic acid; Trans-2-Hydroxycinnamic acid
IUPAC Name	(E)-3-(2-hydroxyphenyl) prop-2-enoic acid
Molecular structure	
Molecular formula	$C_9H_8O_3$
Molecular weight	164.16 g/mol
Chemical Bond	Double-bond stereo
Physical appearance	Crystal white solid

(Source: https://pubchem.ncbi.nlm.nih.gov/compound)

2.2 Drying of *Gliricidia sepium* Leaves

2.2.1 Principle and Mechanism of Thin Layer Drying

Drying of plant materials is an ancient method of preservation as the presence of moistureduring storage may result in microbial growth. The moisture content in leaves needs to be reduced to an acceptable percentage of residual moisture content in order to avoid any reaction and oxidation (Lin *et al*., 2010). In general, drying is a complex operation involvingtransient of heat and mass transfer along with several rate processes such as physical or chemical transformations which in turn may cause changes in mechanism of heat and mass transfer and also product quality (Xiao and Aditya, 2013).

In general, drying process is governed by principle of heat and mass transfer. The moist product is heated to an appropriate temperature either by conduction or convection using external drying medium such as hot air or steam or radiation. In the process, the moisture gets vaporized and diffuses into the environment. The process of moisture removal involves two basic phenomena; vaporization of moisture from the material surface and movement of moisture from the material interior to its surface as a result of diffusion, cell contraction and vapour pressure gradient (Xiao and Aditya, 2013).

Thin layer drying is widely used method to determine the drying characteristics of leafy materials (Onwude *et al.,* 2016). According to American Society of Agricultural and Biological Engineers (ASABE Standard 2001), thin layer drying is defined as drying of a material layer which fully exposed to an airstream during drying process and the layer thickness should be uniform and should not exceed three layers of particles.

In the study, the term “thin layer” has been applied to a single layer of *G. sepium* leaf suspended in the drying air. A multilayer of certain leaf thicknesses can be considered if temperature and relative humidity of the drying air remain in the same thermodynamic state at any drying time. It means that, the thickness of a thin layer can increase if the velocity of the drying air increases and also if the thermodynamic state of the drying air approaches the equilibrium state in heat and mass transfer within the dried leaf layer (Kucuk, 2014).

Figure 2.2 shows a schematic of leaf layers along the drying tray. The layer thickness should be uniform and should be less than 3 layers of particles with an assumption that the temperature distribution of a thin-layer material is uniform. This concept also can be applied to a single material freely exposed to the drying air and a multilayer of different slice thicknesses, provided the drying temperature and the relative humidity of the drying air are in the same thermodynamic condition at any time of the drying process, which thuscan be applied to the mathematical estimations of the drying kinetics (Daniel *et al.,* 2016).

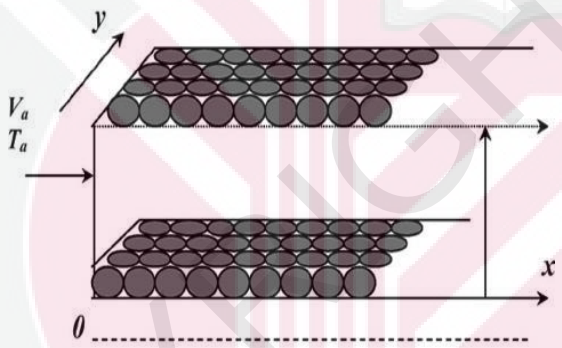


Figure 2.2 : Schematic diagram of thin layer of leaf layers along the drying tray
(Source: Mabrouk *et al.,* 2012)

The leaf drying mainly occurs in two stages; constant rate evaporation period followed bya falling rate evaporation period after a preheating period as shown in Figure 2.3. Both stages show great influence on the whole drying operation. The evaporation takes place at the outer leaf surfaces for the removal of unbound moisture from the outer surface for the removal of unbound moisture from the leaf surfaces (Mabrouk *et al.*, 2012). However,this period is not seen in the leaf drying as unbound moisture in leaf is insignificant. The constant rate

period ends at critical moisture content.

Then, second falling rate period takes place until desired moisture percentage is achieved. During first and second falling rates, the drying rate decreases as the inner moisture content is slowly transported to the outer surfaces before it is fully evaporated from the surface. Even though the quantity of moisture removal during falling rate period is low, the time taken is considerably high makes it as a significant factor in the overall drying process. Hence, the falling rate period has great impact on total drying time as it is dependent on particle size, thickness and external conditions such as temperature, relative humidity and air velocity rate (Chanpet *et al.* 2020). Drying operation stops when it reaches the equilibrium moisture content.

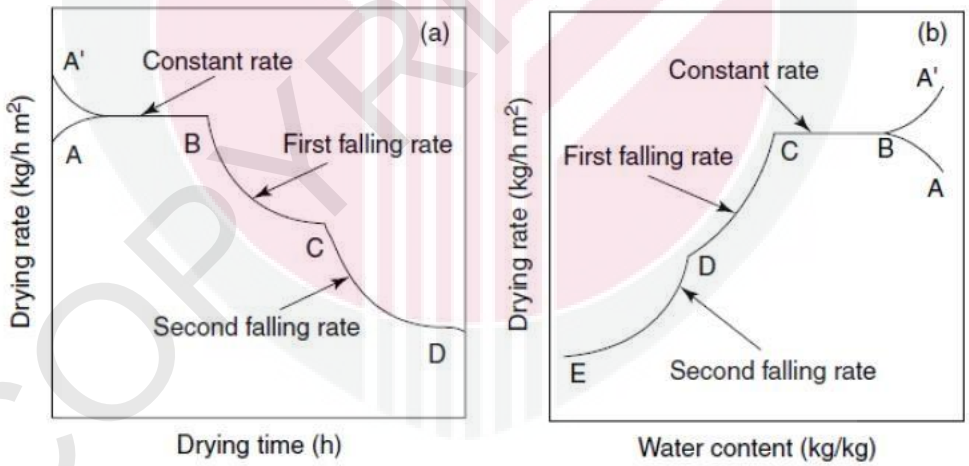


Figure 2.3 : Typical drying curve (Geankoplis *et al.,* 2013)

2.2.2 Classification of Leaf Drying Methods

Leaves can be dried by several methods including thermal drying, chemical drying and other special assisted drying equipment. All the drying methods

show an obviously remarkable in yield volumes. There are several well-known leaf drying methods such as sun drying, shade drying, freeze drying and hot-air drying. Among these drying methods, hot-air oven drying in the temperature range of 40–60 °C is the most common drying method used in leaf drying studies in lab scale experiments (Grant *et al.* 2021).

Shang *et al.*, (2018) highlighted those different drying methods had significant effects on the physicochemical properties of *Symphytum officinale L.* root such as the chemical composition (contents of total polysaccharides and uronic acid), relative viscosity, solubilityand molecular weight. Among hot air drying, vacuum drying and freeze-drying method, the best method to keep the antioxidant activities with maximum polysaccharides yield of 22.87% by using freeze drying method. The authors also claimed that freeze drying method showed stronger reducing power and radical scavenging capacities than the rest methods.

Przemyslaw *et al.* (2018) stated that drying kinetics, bioactive compounds, antioxidant activity and the main sensory parameters determined in dried quinces were affected by the drying method. The highest total polyphenols content was observed in dried samples obtained after freeze drying and convective drying at 50°C. They added that the best drying treatment, considering only the sensory attributes, was vacuum-microwave drying at 480 W, because it led to intermediate dark colour and high intensities of basic tastes and key flavour attributes.

Chuyen *et al.* (2017) claimed that drying methods and drying temperatures significantly affected the drying time, carotenoid content and antioxidant capacity of the dried Gac *(Momordica cochinchinensis S*.) peel. Among the investigated drying methods, hot-air drying at 80˚C and vacuum drying at 50˚C produced dried Gac peel that exhibited the highest retention of carotenoids and the strongest antioxidant capacity.

Rwubatse *et al.* (2012) also stated that drying methods affected functional and pasting properties of sweet orange fruit (*Citrus sinensis, L.)* peel flour/wheat flour blends. The sun-dried orange-peel flour showed higher water and oil absorption capacities than the oven and solar dried of orange peel flours. The water and oil absorption capacities increased with increase in the level of orange fruit peel flours in the blends. The results showed thatsimple and cheap drying methods could supplement wheat flour to improve the functionaland pasting properties during food preparation. For this study, oven drying of *G. sepium* leaves was chosen because of the uniformity in the dried sample. Although, drying methods affect the morphology of the products being dried, whereas, studies have shown that oven air temperature below 80 °C caused no significant loss of plant nutrients (Alara *et al.,* 2019).

2.2.3 Parameters Influencing Leaves Drying

Drying process is mainly affected by several parameters including drying temperature, velocity of drying air, humidity of drying air and product characteristics. The effect of drying temperature is the most widely studied parameter. Generally, the increase in drying temperature shortens the drying

time by elevating the drying rate. However, the use of high temperature is often conceded by the degradation of the product quality. The review on the operating parameters of leaves drying can be seen in Table 2.3.

According to Calin-Sanchez *et al.* (2020), drying as a thermo-physical and physicochemical operation in which excess moisture is removed; hence the water activity of the wet material is reduced. Structure of the wet material, characteristics of the air used, mechanism of moisture migration, types of drying method and dryer used are some of the other parameters which determines the characteristics of the dried material. Inadequate drying not only deteriorates the quality of the yield but also lowers profit from trading of such products. Hence, selection of a proper dryer and drying technique are of utmost importance to achieve commercially feasible desired quality product. Further, performance of the dryer depends on different characteristics of air, product and equipment.

Table 2.3 : Operating Parameters of Leaves Drying

Leaves	Drying methods	Initial MC (g water/g dry matter)	Final MC (g water/g dry matter)	Drying temperature (°C)	Microwave power (W)	Drying air velocity, m/s	Drying time, h	Drying period	References
Kaffir lime leaves	Microwave				264	-	2	Falling rate	Heri *et al.* (2023)
Stevia	Microwave			105	720		-	Falling rate	Baldev *et al.* (2023)
Amaranth Leaves	Microwave vacuum	-	0.043		700	-	0.192	Falling rate	Mujaffar and Loy (2016)
Stevia	Sun and shade	-	-	29.7	-	-	96	Falling rate	Moguel *et al.* (2015)
Mint	Vibro-fluidized bed	-	-	40-60	-	2	4	Falling rate	Ardestani *et al.* (2014)
Moringa	Oven	0.81	0.0477-0.0302	40-70	-	3	2-8	Falling rate	Ali *et al.* (2014)
Papaya	Hot air	-	-	25-43	-	-	30-50	Falling rate	Meijun *et al.* (2014)
Chamomile	Microwave	0.83	-	50	400	0.5	0.5	Falling rate	Motevali *et al.* (2014)

2.2.4 Thin Layer Drying Models

Many models have been used to describe the drying process for different agricultural products. These models are used to estimate drying time of several products under different drying conditions, the way to increase the drying process efficiency and also to generalize drying curves for the design and operation of dryers. Numerous mathematical models for thin layer drying of many agricultural products have been proposed. In the development of thin layer models of agricultural products, the researchers generally measure the moisture content of the product at a known time after it has been subjected to known and constant drying conditions. There are different types of model equations has been developed and applied by many scholars, which are theoretical, semi-theoretical, and empirical models (Ertekin and Firat, 2015).

In general, drying models are usually referred to as models that describe the mass and heat transport within the material being dried as the exterior conditions are already coveredby the theories of environmental heat, mass transfer and momentum transfer (Xiao and Aditya, 2013). The theoretical was developed based on the fundamental physics (mass, energy, and momentum), and the model equation was primarily developed using Fick's second law of diffusion (Benseddik *et al.,* 2018). The models depend on the internal and external resistance to moisture transfer, making the application of this model more complicated in different engineering applications.

The semi-theoretical was modified from the theoretical, which is more simplified derivationfrom Newton's cooling laws (Kucuk *et al.,* 2014). The models require

the use of experimental data with fewer assumptions.

While for the empirical models neglect the fundamental aspects of drying, and the model parameters are strongly depending on the experimental data of drying and the dimensionless analysis (Onwude *et al.,* 2016; Inyang *et al.,* 2018; Kucuk *et al.,* 2014). The drying temperature, air velocity, material thickness, initial moisture content, and relative humidity are among the externally influenced empirical model (Onwude *et al.,* 2016). According to the literature, thin layer drying models using empirical offered more accurate results and better prediction of drying characteristics in fruits and vegetables than designed thin layer drying models (Inyang *et al.,* 2018).

The diffusion model assumes that mass transfer within the material is governed by Fick's second law of diffusion. The differential equations for three types of shapes are given as below (Benseddik *et al.,* 2018):

For sphere, $$\frac{\partial W}{\partial t} = D_e\left(\frac{\partial^2 W}{\partial r^2} + \frac{2}{r}\frac{\partial W}{\partial r}\right) \quad (2.4)$$

For cylinder, $$\frac{\partial W}{\partial t} = \frac{D_e}{r}\frac{\partial}{\partial r}\left(r\frac{\partial W}{\partial r}\right) \quad (2.5)$$

For slab, $$\frac{\partial W}{\partial t} = D_e\frac{\partial^2 W}{\partial r^2} \quad (2.6)$$

where W is weight of water within product (kg), D_e is the effective diffusivity coefficient (m^2s^{-1}) and r is the distance form centre of axis. The effect of sample temperature and local moisture content on the effective diffusivity is taken into

account by using Arrhenius Equation 2.7 (Darvishi, 2017).

$$D_e = A\exp\left(-\frac{E_a}{RT}\right) \tag{2.7}$$

Where;

A= pre-exponential factor of Arrhenius equation (m^2s^{-1})

E_a = energy of activation (J/mol)

R = universal gas constant (8.314 J/molK)

T = temperature (K).

The thin layer models have been applied to describe drying process of various products and drying methods. There is, in fact, no universal model among both types of models that can fit all the data. The suitability of the model is strongly dependent on the characteristics of product and drying technique. The moisture removal processes and their relationship on the process variables are expressed in terms of drying kinetics. Thus, the determinationof the drying rates is required for the development of reliable process model.

Table 2.4 lists the results of the mathematical modelling of drying process of several products. Comparing to the similarity of size and structure, *G. sepium* leaves are mostly similiar to the bay leaves which best suited with the Midilli and Kucuk models (Ghinimi *et al.,* 2016).

Table 2.4 : The best thin layer models of drying for different leaves

Best Model	Drying Kinetics Equation	Leaves	Drying methods	References
Lewis	$MR = exp(-kt)$	Mate	Fixed bed	Shinde *et al.* (2013)
		Tea	Batch type	Panchariya *et al.* (2002)
Page	$MR = exp(-kt^n)$	Thymus	Solar	El-Sebaii *et al.* (2012)
		Pandanus	Microwave	Rayaguru & Routray (2011)
		Olive	Solar	Boudhrioua *et al.* (2008)
		Moringa	Oven	Ali *et al.* (2014)
		Rosemary	Oven	Bensebia *et al.* (2015)
Modified Page	$MR = exp(-kt)^n$	Thymus	Solar	El-Sebaii *et al.* (2012)
		Rosemary	Microwave	Arslan *et al.* (2008)
		Kaffir lime	Heat pump-dehumidified	Trirattanapikul *et al.* (2014)
Henderson & Pabis	$MR = a\ exp(-kt)$	Mint	Hot air	Costa et al. (2014)
Two-term	$MR = a\ exp(-k_1t) + b\ exp(-k_2t)$	Tea	Withering	Anindita & Partha (2018)
Logarithmic	$MR = a\ exp(-kt) + b$	Mint	Cabinet	Hussein *et al.* (2016)
		Rosemary	Sun and oven	Arslan *et al.* (2008)
		Chilli	Hot air & infrared	Mihindukulasuriya *et al.* (2015)
Midilli and Kucuk	$MR = a\ exp(-kt^n) + bt$	Mint	Microwave	Onwude *et al.* (2016)
		Bay	Oven	Doymaz (2014)
		Rosemary	Sun and oven	Arslan *et al.* (2008)
		Parsley	Air	Doymaz *et al.* (2006)
Wang & Singh	$MR = 1 + at + bt^2$	Banana slices	Solar, sun	Fadhel *et al*. (2011)

2.2.5 Effective Moisture Diffusivity

The moisture migration process during drying is very complex and often involves one ormore transport mechanisms such as liquid diffusion, vapour diffusion, surface diffusion, and hydrostatic pressure differences (Amira *et. al,* 2014). Drying rate increases as a result of equilibrium concentration of the water vapour on the surface of the materials at higher temperature. This produces a migration of moisture from the solid to the surface, which occurs through one or more mechanisms, namely, molecular diffusion, capillary flow, *knudsen* flow, hydrodynamic flow, or surface diffusion. As a result, analysis of the mass transfer phenomenon is based on the assumption that effective moisture diffusivityrepresents all parameters influencing the process rate.

The difficulties of applying effective moisture diffusivity theory to drying processes arise from the complex physical structure and composition of materials. One of the undesirablechanges which occur simultaneously within moisture diffusion in drying process is the volume reduction or shrinkage, physical properties, heat and mass exchange area and in particular affecting the diffusion coefficient of the materials. In general, shrinkage occurs as a result of volume reduction due to evaporation of the moisture contained in the solid. Heating and loss of water cause stresses in the cellular structure of the food and lead to changes in shape and decrease in dimensions (Mayor & Sereno, 2004).

Fruits and vegetables have high initial moisture contents (80–90%) and suffer alterationsto their original form during the drying process due to significant

shrinkage. Generally, different types of drying methods give different value of effective moisture diffusivity. Table 2.5 summarizes various effective diffusivity values among various materials to be dried. Based on the table, vacuum drying of eggplant shows that at 50˚C effective moisture diffusivity value is highest compare to heat pump drying of banana at the sametemperature. This indicates that effective diffusivity is not dependent to temperature only,but it depends on the type of drying process and the materials to be dried.

Table 2.5 : Effective moisture diffusivity values among various plant materials

Plant Materials	Drying Method	Temp (˚C)	Effective Diffusivity, Deff (m^2/s)	References
Clove leaves	Microwave	60	9.9×10^{-11}	Heri *et al.* (2023)
Stevia	Microwave	50	1.99×10^{-10}	Baldev *et al.* (2023)
Myrtle	Convective	50	13.06×10^{-10}	Marwa *et al.* (2022)
Banana	Convective	60	8.08×10^{-10}	Turkan *et al*. (2020)
Prickly pear	Infrared	60	2.53×10^{-10}	Amira *et al*. (2014)
Cassava	Convective	95	1.59×10^{-9}	Fernando *et al*. (2011)
Pumpkin	Convective	95	1.37×10^{-9}	Fernando *et al.* (2011)

2.3 Solid Liquid Extraction of *Gliricidia sepium* Leaves

2.3.1 Mechanism and Mass Transfer of Solid Liquid Extraction

Solid liquid extraction involves the transfer of a soluble fraction (solute) from a solid material to a liquid solvent. The solute diffuses from the solid into the surrounding solvent.This term may also be considered as dissolving of one or more components in a solid matrix in simple solution or by the formation of a soluble form by chemical reaction (Chanioti, 2014).

Solid liquid extraction is similar to liquid-liquid extraction except that thesolute initially resides in a solid. Fundamentally, solid liquid extraction involves several phases as shown in Figure 2.4.

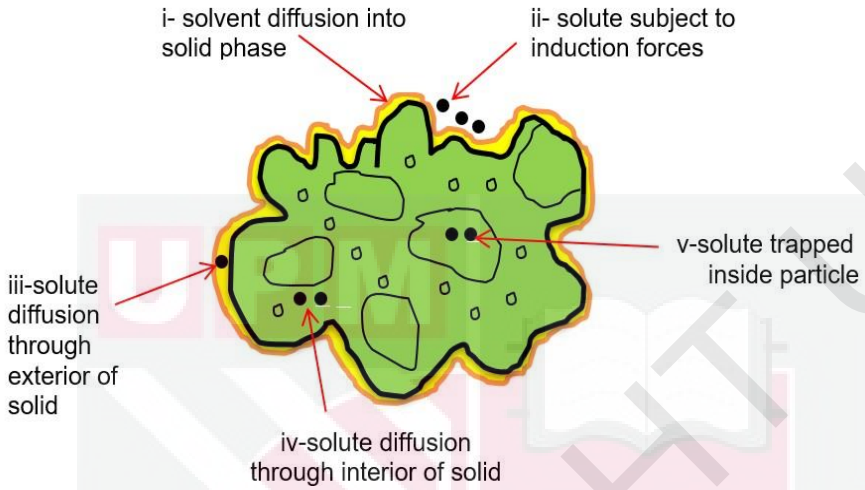


Figure 2.4 : Mechanism of bioactive compounds exraction in SLE

The solvent initially diffused into the solid phase. Then, the solute changes its phase andstarts to dissolve in the solvent. The solutes then diffuse through the exterior of solid before internally diffuse into the interior of solid. The solute is trapped inside the solid particle. The extracted solute is finally transferred from the external surface of the solid particle into the main bulk of the solution.

From an engineering point of view, solid liquid extraction is a multiphase, multicomponentand involves unsteady state of mass transfer operation. Mass transfer is defined as migration of a system component from phase to phase or in the same phase due to concentration gradient between two points. The mass transfer is carried out by molecularflow due to molecular diffusion. In diffusion, the concentration gradient is the cause of the flow and the heat

gradient is the driving force. The diffusion phenomenon occurs in all mass transfer operation which involve at least one phase and usually in both phases (Ibarz and Barbosa-Canovas, 2019).

In solid liquid extraction, the solute diffuses through the solid phase into the liquid. The solvent fills the solid pores and remains in stationary mode in contact with the solute within the solid. The solute is diffused to the solvent by molecular diffusion. In this stage,the mass transfer rate is in accordance with the Fick's law equation as follow (Ibarz and Barbosa-Canovas, 2019).

$$N_s = -D_L \frac{dC}{dz} \tag{2.8}$$

(m^2/s), C is the solute concentration and z is the distance inside the pore (m). The masstransfer and the diffusivity are further can be increased by increasing the temperature. The mass transfer rate also can be increased by crushing particles which will result in decreasing of pores length. Due to the concentration gradient, the solute is transferred into the solution as it reaches the surface of the particle. At this stage, the mass transferis carried out by molecular and turbulent transport concurrently (Ibarz and Barbosa- Canovas, 2019) which can be expressed as;

$$N_s = \frac{dM}{Adt} = K_L\left(C_s - C\right) \tag{2.9}$$

Where M is the mass of transferred solute (kg), A is the particle-solution contact surface(m^2), t is the time (s), C_s is the solution concentration at the solid surface (kg/m^3), C is thesolute concentration in the solution at a determined

instant (kg/m^3) and K_L is the mass transfer coefficient (m/s).

The mass transfer proceeds until it reaches the equilibrium. Equilibrium is a state in whichthe solute is entirely dissolved, thus the concentration of solution remains uniform over the time, providing enough solvent volume for total dissolving of solute and the solute isnot absorbed by the solid. Generally, there are two phases involved in an equilibrium reached system. The one is overflow, which means separated solution also known as miscella and the another is underflow which means the solid residue along with the solution remain inside them (Ibarz and Barbosa-Canovas, 2019).

2.3.2 Solid Liquid Extraction Modes of Operation

The solid liquid extraction processes can be batch or continuous (Sofia *et al,* 2014). A batch extraction system consists of an agitated mixing vessel where the solids are treated with the solvent by percolation or immersion. Batch extraction is carried out on certain cases such as the extraction of pigments from plants, the isolation of proteins from oil seeds and so on. Batch extractors are easy and simple but limited capacity anddiscontinuous output of the extract are its main drawbacks (Sofia *et al.,* 2014).

The second one is continuous multistage extraction systems in which solids are moving from one stage to another continuously. Moving-bed percolation extraction equipment isan example as shown in Figure 2.5 commonly used for treatment of various vegetable seeds such as cottonseed, rice bran, soybeans, peanuts, and castor beans. The pretreatment of the seeds is

required and consists of dehulling, precooking the adjustment of water content and forming into flakes or rolls and increasing the specific surface exposed to the solvent. The advantages of this extractor are that the miscella isfree of solids since self-filtration takes place, the residue can be well drained when the equipment is controlled and large amounts of solids can be treated continuously (Sofia *et al.,* 2014; Rao, 2010)

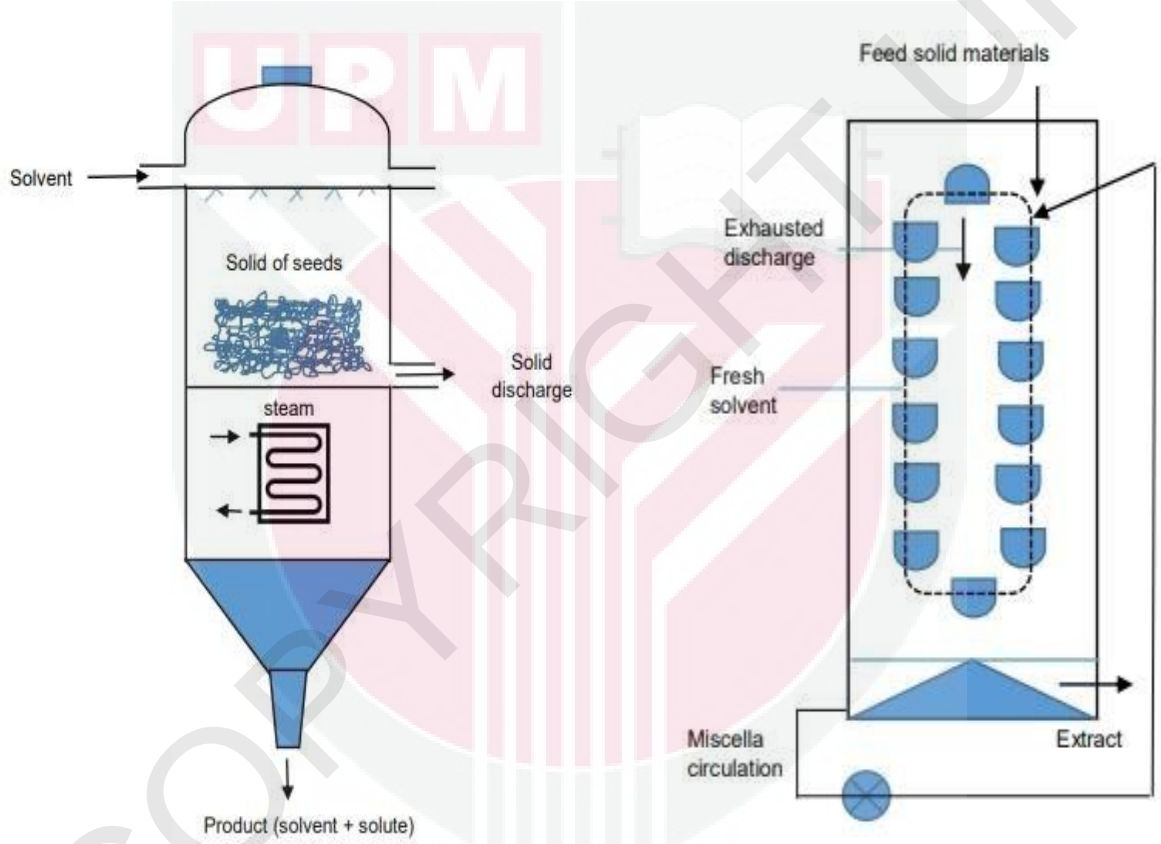


Figure 2.5 : Batch and continuous diagrams of SLE extractors
(Source: Sofia *et al.,* 2014)

2.3.3 Solid Liquid Extraction Methods

There are numerous extraction techniques available, with the option to choose betweenconventional and assisted extraction techniques (Azmin *et al.,* 2023). The commonly used traditional methods are maceration, decoction, soxhlet,

percolation and infusion. Meanwhile, the assisted extraction techniques are widely proposed as an alternative to resolve the issue of lower efficiency and quality than conventional approach (Tsaltaki *et al.,* 2019; Arruda *et al.,* 2019). The main characteristics of these alternatively assisted extraction techniques and the traditional methods are comparatively summarized in Table 2.6 below:

Table 2.6 : Comparative features of assisted and traditional extraction techniques

Extraction Methods	Features		
	Driving force	**Advantages**	**Disadvantages**
Assisted ExtractionTechniques			
Ultrasonic- Assisted	Acoustic cavitation	Simple apparatus setup solvent safe, reproducible	Possible compound degradation due to high frequency
Microwave-Assisted	Microwave power	Moderate equipment cost, less solvent usage and simple operation	Possible compound degradation due to heat effect
Supercritical Fluid	Pressure and supercritical fluid	Use no hazardous solvents, low temperature set	Require higher setup cost, involve lots of parameters optimisation
Traditional Extraction Techniques			
Maceration	Solvent contact	Easy apparatus set up	Higher solvent volume, long processing time, less recovery
Soxhlet	Heat	Simple instrument set up	Possible degradation of thermolibe compounds, long processing time
Mechanical agitation	Speed and solvent contact	Simple instrument set up	Possible solvent vaporisation, higher solvent volume, long processing time, less recovery

The assisted extraction process offers multiple advantages such as high recovery, a fastextraction rate, protected thermosensitive compounds and a green solution method (Saifullah *et al.,* 2020; Tradit *et al.,* 2011; Deng *et al.,* 2017; Putnik *et al.,* 2018). The ultrasonic assisted, microwave-assisted and supercritical fluid extractions are among themost selected assisted extraction techniques that commonly used in plant extraction (Wen *et al.,* 2020).

2.3.3.1 Soxhlet Extraction

According to Wei *et al.,* (2013), they extracted ursolic acid from the *Cynomorii Herba* witha yield of 38.21 mg/g by Soxhlet extraction. They further concluded that the applied SE was able to achieve desirable yield with optimum extraction conditions as ethanol solvent of 95%, ratio of materials to solvent of 1:18 (g/mL), the extraction temperature of 90°C for 2 hours. Among other conventional methods, SE offers simple operation set up but requires higher processing time, thus requires high volume of solvent (Emdad *et al*., 2019; Shital *et al.,* 2015 and Elumalai *et al.,* 2016).

Parthiban *et al.,* 2017 also tested on anti inflammatory of various bioactive compounds including flavonoids, glycosides, alkaloids, aleurone and triterpenoids from stem of*Zanthoxylu mrhetsa* using soxhlet extraction with 2-3 liter of solvents (benzene, ethanoland methanol). Kasiramar *et al.,* (2019) added that benefit of using SE was instead ofmany portions of warm solvent being passed through the sample, just one batch of solvent is recycled. However, SE was not suitable for thermolabile compounds as extended heating may lead to degradation of desired compound.

2.3.3.2 Ultrasonic Assisted Extraction

Ultrasonic-assisted extraction (UAE) is a modern extraction method and has been examined for the recovery of various plant extracts. UAE has been used as an alternativefor solvent extraction due to its several advantages such as inexpensive equipment, simplicity and remarkable reduction in smaller solvent consumption, extraction time andtemperature (Bimakr *et al.*, 2012). Among the assisted extraction techniques, UAE offersvaroius advantages such as rapid, economically friendly with simple apparatus, low solvent consumption and less expensive to retain the functionality of the phenolic compounds (Chemat *et al.,* 2017; Kumar *et al.,* 2021; Mahdi *et al.,* 2019, Farid *et al*., 2020). The mechanical impact of ultrasonic speeds up the releases of bioactive compound from plant material extraction process by permitting a higher diffusion rate ofsolvent into the cell membrane (Pollini *et al.* 2020). In addition, the extraction process could be done at a lower temperature which is more suitable for the thermo-labile compounds (Saifullah *et al.,* 2020).

Besides, several studies proved that this economical method could improve the extractionquality (Xu *et al.,* 2019; Oroian *et al.,* 2020; Boeira *et al.,* 2018). The main mechanism involved in ultrasonic is acoustic cavitation as shown in Figure 2.6. Upon exposure to the ultrasonic, the sound waves propagate through the liquid medium, facilitate cycles of compression and rarefaction and create cavitation bubbles (Kumar *et al.,* 2021). The series of those cycles create an acoustic pressure, whereby bubbles form and collapse (Dalmau *et al*., 2020). Acoustic cavitation can improve extraction efficiency, resulting in the rupture or breakdown of plant cell walls (Kumar *et al.,* 2021; Bendicho and

Lavilla 2013). In other words, UAE works based on the principle of acoustic cavitation which is capable of damaging the cell walls of the plant matrix and thereby favoring the release ofbioactive compounds (Medina *et al.,* 2017).

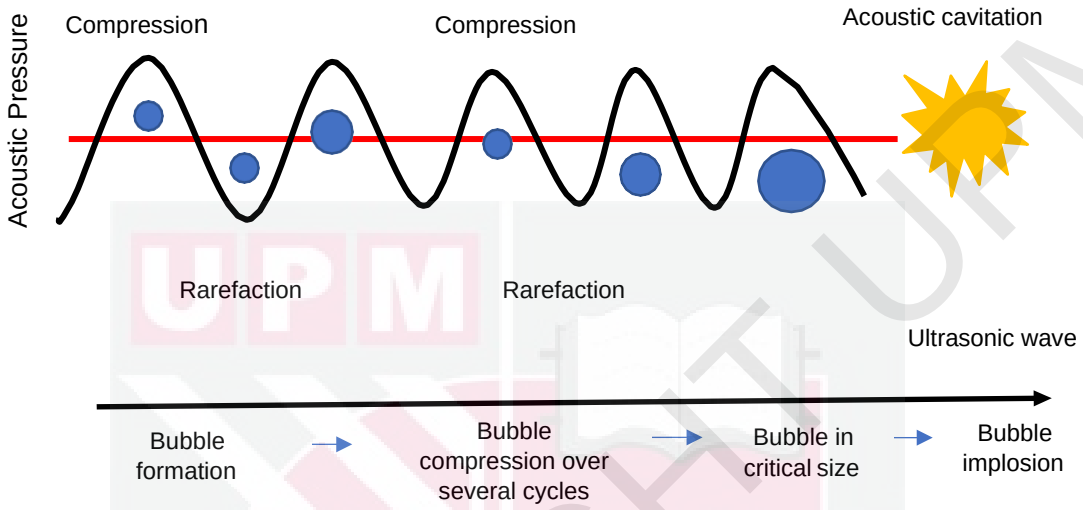


Figure 2.6 : Mechanisme of ultrasonic effect

Different designs are available in UAE and the selection is primarily based on their modesof action such as ultrasonic bath, probe and special-design method (combining design oftwo ways) (Fu *et al.,* 2020; Bendicho and Lavilla 2013). Each device is differently designed and created different sonication effects.

The ultrasonic bath is the most used system which is suitable for cleaning, degassing of liquid or solvent used in HPLC, sample pre-treatment (for breaking the sediment or particle). extraction, and synthesis processes (Sharifalhoseini *et al.,* 2015; Pollini *et al.,* 2020; Dranca and Oroian 2016; Ma *et al.,* 2014). The ultrasonic bath consists of stainlesssteel tank, ultrasonic generator, temperature controller, base tranducer and heaters as shown in Figure 2.7. For operational purposes, samples are immersed inside in a tank

containing water. Water is utilized as a carrier to transmit the ultrasonic energy and the ultrasonic frequency should be adequate to create cavitation in the vessel.

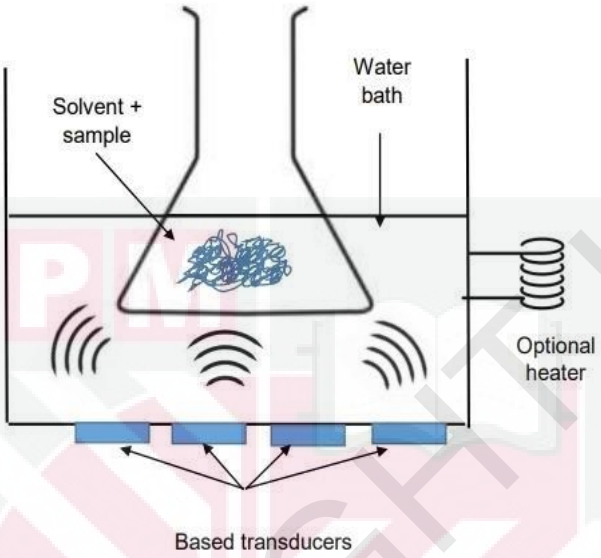


Figure 2.7 : Schematic diagram of ultrasonic bath with indirect system

Several studies discovered that the indirect UAE produces a higher extraction yield than the traditional extraction method. According to Saifullah *et al.,* (2020), employing an ultrasonic bath rather than a conventional shaking water bath increased the recovery of phenolic components and the antioxidant capacity of *Leptospermum petersonii* leaves. The influence of extraction parameters on the extraction efficiency in the indirect UAE was reported in various plant samples.

Oroian *et al.,* (2020) reported the impact of ultrasonic amplitude, ethanol concentration, temperature, and time on the recovery of BC *propolis.* Meanwhile, Vu *et al.,* (2017) investigated the effects of time, temperature, pH, and sample to solvent ratio on the extraction yield of catechins from green tea

leaves.

Second is the ultrasonic probe system which is used as a sample homogenizer. This system consists of a generator, an upper titanium horn element and a detachable horn. The temperature controller or stirrer sometimes are included in the system. The generator controls the input parameters such as time, intensity (represent in %), temperature, and pulsed mode action. The energy was generated from this generator and transferred to the upper horn, which then forms an emitter and boaster. A horn allows acoustic cavitation and consequently creates erosion and bubbles (Tsaltaki *et al.,* 2019; Dumitrascuet *al.,* 2019). Figure 2.8 shows the schematic diagram of direct UAE used in this study (QSonica, Q500).

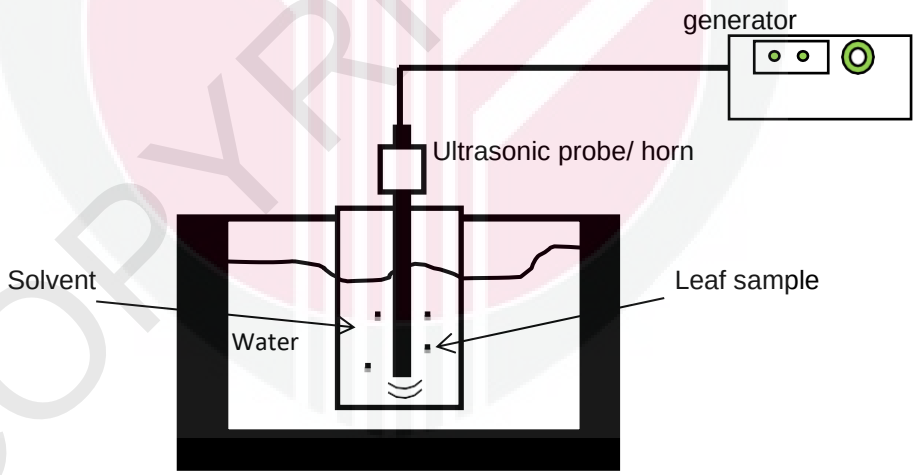


Figure 2.8 : A schematic diagram of direct UAE
(Source: Bikmr, 2012)

The direct UAE is also called the direct sonication method, where the ultrasonic wave willdirectly transfer and touch the samples. The power allows energy to be supplied directly to the sample, which can rapidly raise the solvent's

temperature. A double-walled vessel or an ice bath is occasionally used to manage the temperature by cooling the water inside the sample reservoir and protecting the bioactive components. Despite its high- power generation performance when compared to the indirect UAE, issues such as volatile component loss owing to degassing effects remain (Bendicho and Lavilla 2013).

The cavitation effect in the direct UAE is stronger than in the ondirect UAE due to its high intensity, which generates mechanical stress during the operation (Dash, Pathak, and Pradhan 2021). The direct intensity from the probe's tip on the samples would create more bubbles, collapse near or on its plant surface, and break the cell walls. This effect significantly influences the mass-transfer process to release the extractable components (Martinez-Patino *et al.,* 2019).

Odabas and Koca (2016) previously compared the different extraction assisted methodologies. Impressive findings from their research indicated that extraction coupled with direct sonication would be the optimum technique for phenolic component extraction with enhanced antioxidant content from hazelnut skin. However, the use of high power and frequency can occasionally lead to the oxidation or breakdown of compounds, especially phenolics, which produces extremely reactive radicals (Medina-Torres *et al.,* 2017). This argument was supported by the previous study that the extraction using the direct UAE generated low catechin value, possibly due to the degradation of catechin effect on the sample matrix (Jacotet-Navarro *et al.,* 2015;

Albuquerque *et al.,* 2017). Thedirect UAE was employed to reduce extraction time and solvent consumption.

The positive linear effect of using direct UAE to increase the phenolic extraction was reported in different plants such as Pyrus communis fruit peel (Tomsik *et al.,* 2016), hazelnut skin (Odabas and Koca 2016), Blueberry Wine Pomace (He *et al.,* 2016), olive cake (Mojerlou and Elhamirad 2018), rice grains (Setyaningsih *et al.,* 2019), Melastoma malabathricum leaves (Awang *et al.,* 2021), and Araticum peel (Arruda *et al.,* 2019). Theresults from the studies were achieved in a rapid processing time with the enhanced recovery of phenolic compound and antioxidant activity, for example, 3 mins for olive cake (Mojerlou and Elhamirad 2018), 5 mins for 11 min for Starkrimson fruit peel (Belwal *et al.,* 2019), 15 min from Melastoma malabathricum leaves (Awang *et al.,* 2021) and 25 mins for rice grains (Setyaningsih *et al.,* 2019). This system is preferable compared to indirect UAE requires a longer time, which is more than 30 mins (Oroian et al., 2020; Tomsik *et al.,*2016); Arteaga-Crespo *et al.,* 2020) and Dumitrascu *et al.,* 2019).

The employed direct UAE can reduce the extraction time and solvent usage, with a promising phenolic compound recovery due to the formation of a more considerable cavitation effect. Reviews indicated that the study is currently limited and still not comprehensively focused on the markers or major O-C from *G. sepium* leaves. Thus, acomparative analysis should be developed and proposed to investigate the extraction performance in terms of extraction yield.

2.3.4 Factors Affecting the Efficiency of Ultrasonic Assisted Extraction

2.3.4.1 Solvent Selection

The solvent selection determines the yield of extracted phytochemicals. Solvents are categorized into two groups including polar and non-polar. Polarity of a solvent, expressed in term of dielectric constant, determines the types of compounds to be dissolved. The basic principle in solvent extraction is 'like dissolve like' in which polar solvents extract polar phytochemicals and vice versa. High polarity solvent like water andaqueous solvents are preferably used for the extraction of tannins and polyphenols (Zhang *et al.,* 2018).

Minh *et al*. (2022) claimed that green extracting solvents have great effect on antioxidant, xanthine oxidase and plant inhibitory potentials of solid-based residues of *Cordyceps Militaris.* The solid-based residues of ethanol-water (60:40) and ethanol-water (40:60) exhibit the highest antioxidant activity and optimum content of the yield. Xu *et al*. (2022) reported that the addition of water increased the yield of total-isoflavones from extraction of kudzu vine (*Pueraria lobata*) significantly. The results showed that 50% ethanol aqueous solution gave a four-time higher yield than 100% ethanol while 1:1 (v/v) n-butanol/water two-phase solvent system gave a seven-time higher yield than n-butanol.

According to Masturah *et al*. (2019), the yield of *Phyllanthis niruri* extraction using Soxhlet method in the increasing of order was hexane > acetone > chloroform > ethanol > methanol > water. The results revealed that the solvent

with higher polarity gave higher yield. The study also showed that components with carboxylic acid and hydroxyl groups are preferably extracted by water through hydrogen bonding. Rakotondramasy- Rabesiaka *et al*., (2018) also added, 50% ethanol aqueous solution was also the most effective solvent for extraction of protopine from *Fumaria officinalis* L. compared to water and 95% ethanol. The use of aqueous mixture as solvent was found to be more effective than single-compound solvents in other extraction systems. The aqueous mixture could extract both polar and less polar phytochemicals and water helps in penetrating the cell wall and membrane. The other literatures on solvent system in plant extraction were summarized in Table 2.7.

Table 2.7 : Solvent system for various extraction systems

Extraction Plants	Bioactive Compound	Solvent Extraction	Optimum Yield	Conclusion	References
Powdered apple pomace	Quercetin, phloridzin, phloretin, catechin, epicatechin, procyanidin	30% ethanol-water	30% quercetin as main compoundrecovered	Extraction efficiency was higher in70% ethanol using UAE	Rashid *et al.(*2023)
Ginger leaves	Phenolic compounds (gingerols and shogaols)	Methanol, ethanoland water	Out of seven species, 95% methanoland 95% ethanol showed high inhibitory and cytoxicity activity	Ginger possesses antioxidant, anti-inflammatory, and antimicrobial properties preferably in methanolic solvent	Kamarudin *et al.* (2023)
Agarwood	Resin and essential oil	Methanol, ethanoland water	All 11 compounds have been isolatedfrom crude methanolic extract	Methanol and ethanol were mostly used in agarwood extraction thanwater due to impurities	Zakaria *et al*.(2020)
Curry leaf, torch gingerand lemon grass	Rutin, quercetin-3-glycoside, myrecitin, quercetin, caffeic acid, p-coumaric acid and curcumin	80% acetone, 80% ethanol, 80% methanol usingUAE	The recovery of phenolic compoundsfrom curry leaf (92.23 mg GA/g freeze-dried crude extract)	80% ethanol was the mostappropriate solvent.	Sepahpour *etal*. (2018)
Honey bract	Caffeic, ferulic, benzoicacids, rutin, quercetin, kaempferol	Methanol usingsolid phase extraction	TPC in honey samples varied from 71.7 to 202.6 µg/g with the highest indark honey.	Phenolic acids prevailed in most samples using methanolic solvent	Arroy *et al.(*2017)
Dragon fruitpeel	Betacyanins	10% ethanol – water using SFE	The peel and flesh extracts contained 24.58 and 91.27mg/100ml totalbetacyanin	The flesh extract had the stronger antioxidant activity than the peelextract	Fathordoobady *et al.* (2016)

2.3.4.2 Ratio of Solvent to Solid

The amount of solvent used in the extraction is normally expressed in ratio to the amountof solid (R_{ss}). The increase in the amount of solvent increases the rate of extraction because the mass transfer is driven by the concentration gradient between the boundarylayer. It also raises the yield by elevating the degree of saturation. The greater value of mass ratio of ground solvent to solid, the greater the driving force of mass transfer.

Aung *et al.* (2023) claimed that the solvent to solid ratio should be higher for rhein extraction *from Senna alata* leaf extract. They found that solvent to solid ratio of 25:1 (mL/g) was optimum at 10.44 mg/g extract. According to Sinaga *et al.,* (2018), the extraction of glycerol by the solvent ethylene glycol gave the highest purity up to 90.646%at the ratio of solvent to solid of 15: 1 (mL/g) with extraction time 60 minutes. They addedthat, the results indicated that the more volume of solvent used, the less extraction time to produce high purity of glycerol. Silva *et al*. (2017) also reported that the higher ratio ofsolvent to solid resulted in higher yield in extraction of phenolics from *Inga edulis* leavesbut the increase was marginal when the ratio was greater than 20:1 (ml: g). A similar result was reported in the study of extraction of protopine from *Fumaria officinalis* L. (Rakotondramasy-Rabesiaka *et al*., 2019). The results imply that the yield is affected bythe total solute available within the raw material. The other literatures on solvent to solidratio in plant extraction were summarized in Table 2.8.

Table 2.8 : Ratio of solvent to solid (Rss) values using UAE systems

Extraction Plant (s)	Bioactive Compound	Extraction solvents	Rss (ml/g)	Optimum Yield	Conclusion	References
Greek sage	TPC, TFC and rosmarinic acid,carnosol and carnosic acid	Ethanol	10:1	The optimal processing parameters were obtainedusing 68% ethanol	The higher ethanol concentration with lower extraction temperature arerequired for optimum extracts	Irakli *et al*. (2023)
Dried olive leaves	Phenolic compounds	80% ethanol hydroalcoholic mixture	20:1	The highest content of oleuropein and luteolin-7-O-glucoside was determined as31 ± 2 and 4.1 ± 0.2 mg/g dried olive oil.	Faster extraction was observed by UAE, althoughsimilar final extraction yieldwas reached by both technologies.	Kashaninejad *et al. (*2020)
Olive oil	Phenolic compounds	80% ethanol	30:1	Total phenolics (24.5mg caffeic acid/g dry matter)	The higher of TPC recovered with increasingethanol concentration, temperature and Rss	Noureddine, 2018
Banana peel	Vitamin C	Methanol	15:1	The highest yield was 0.04939 ± 0.00080 mg ascorbic acid	Higher yield with increasingsolvent to solid ratio but a slight decrease when the temperature was increased above 60ºC	Mastor *et al.* (2016)
Dukung Anak	Phenolic compounds	60% ethanol: water	20:1	About 5788.7 mg GAE/100 gDW and 1906.5 mg CE/100 g DW with high antioxidant capacities	TPC decreased with increasing sonication time, and decreased slightly with increasing solvent amount.	Wong, Tan and Ho,(2013)

2.3.4.3 Extraction Time

The process duration of exraction process using SE and UAE is determined based on the equilibrium time which can be estimated from the kinetics data. After the equilibriumis achieved, the increase of the solute concentration in the following time is low and negligible. Thus, it would be a waste of energy to continue the process and thermal degradation of the phytochemicals might take place. The time for achieving the equilibrium state depends on the characteristics of extraction system such type of solid, type of solvent, extraction temperature and so on. It was also reported that an extended exposure to ultrasonic waves did not lead to further improvements in the extraction efficiency.

Belwal *et al.,* (2019) discovered that the extraction yield increased from 7 to 20 mins, however further increasing the time reduced the extraction yield. Yang *et al.,* (2017) observed that the phenolic results declined as the time reached at 60 mins. This conditionis possibly due to the decomposition of phenolics. Oroian *et al.,* (2020) reported that increasing the ultrasonic time from 15 to 45 mins did not affect the targeted yield. It was also discovered that the diameter of propolis. Table 2.9 shows the summary of extractiontime values from other reseach using various ultrasonic extraction systems.

Table 2.9 : Various extraction times using UAE system

Extraction Plant	Bioactive Compound	Extraction Time (min)	Optimum Yield	Conclusion	References
Stephania roots	Alkaloids: Fangchinolinee(FAN), Tetrandrine (TET) and Tetradium ruticarpum (TR)	52	Optimum of alkaloids were attained 7.23, 13.36, 20.59 mg/g, respectively with extraction temperature of 52 °C	Higher extraction time lower the alkaloid yield	He *et al.* (2023)
Olive cake extract	Total phenolic compounds (Protocatechuic acid, cinnamic, ferulic, sinapicacid etc)	30	The TPC content and antioxidant activity were measured 4.04 mg/g and 68.9%, respectively.	The TPC content was slightly increased by lengthen the extraction time	Mojerlou & Elhamirad (2018)
Lemon balm	Rosmarinic acid	33	The highest yield of rosmaniric acid was 31.29%	Longer extraction time showed the decreasing yield pattern	Caleja *et al.* (2017)
Pumpkins and peaches	Total phenolic compounds	28	The optimum yield of peach extract was 43.99%extraction temperature of41 °C	UAE processes were significantly better than solvent extractions with ultrasonic effect	Altemimi *et al.* (2016)

2.3.4.4 Extraction Temperature

Generally, increasing the extraction temperature during solid liquid extraction leads to the increase of extraction yield by enhancing the solubility of phytochemicals and the rate ofmass transfer. The increase of temperature from 30 to 50ºC increased the equilibrium yield of coffee oil by about 8% (Gligor *et al.,* 2023). The rise of temperature causes a decrease in viscosity and surface tension, as well as an increase in vapor pressure, whichcontributes to the extraction efficiency (Chemat *et al.,* 2017).

Additionally, this is in accordance with the equilibrium principle, which states that a higherextraction temperature will result in a faster extraction rate and a shorter extraction timefor a maximal extraction recovery (Zhou *et al.,* 2013). The rise of temperature will aso increase the number of cavitation bubbles and surface contact area, as well as decrease the viscosity and density of the solvent media. These factors governed the higher releaseof phenolics from the plant material, simultaneously breaking the internal plant cells, andincreasing the solubility and diffusion coefficients (Kumar *et al.,* 2021).

Based on the findings of Jayakumar *et al.* (2023), the yield of targeted andrographolide from extraction of *Andrographis paniculata* increased when extraction temperature was raised from 40 to 60ºC. Bucic-Kojic *et al.* (2019) also found a positive effect oftemperature on the SLE of total polyphenols from grape seeds with the highest yield wasobtained at 80ºC. An increase of yield with increase of temperature from 15 to 75ºC wasreported in the extraction of protopine from from *Fumaria officinalis L*. (Rakotondramasy-Rabesiaka *et al.,*

2019). The yield of total phenolics extracted from *Inga edulis* leaves also indicated an increasing trend with the rise of temperature (Silva *et al.,* 2017). Ho *et al.* (2015) demonstrated that the increase of temperature resulted in the increase of extraction rate and equilibrium in extraction of water-soluble compounds from Tilia sapwood.

In addition, Cacace & Mazza (2019) also reported that there was a sharp decrease in anthocyanins at temperatures higher than 45^{o}C in its extraction from milled frozen blackcurrants. These results indicated that higher extraction temperature might give greater total yield but also destroy the heat-sensitive phytochemicals which determine the efficacy of the extract. Hence, the effect of extraction temperature must be carefully studied in order to leach out the desired phytochemicals with maximum rate and quantity. Other literatures were also stated in Table 2.10 with other various extraction temperatures.

Table 2.10 : Various extraction temperatures using UAE system

Extraction Plant	Bioactive Compound	Extraction Temperature (°C)	Optimum Yield	Conclusion	References
Jew's mallow	Flavanoids	68	The highest yield of total flavonoids (7.17 mg QE/g DW) and total polyphenol content (13.92 mg GAE/g DW) were recorded	The developed could contribute to the good recoveryof natural antioxidants from *C. olitorius.*	Biswas *etal.* (2024)
Black cumin	Polyphenols	69	The optimum yield and antioxidant activity obtained were 34.53% with enhanced antioxidant activity, having IC_{50} value of 203.56 µg/mL.	The antioxidant activityshowing a decreased trend from 295 to 203 µg/mL with increase in extraction temperature from 40 to 60 °C.	Dar *et al*. (2024)
Korean apple peel	Total phenolic compounds	20	The optimized extraction conditions that maximized 35% of extraction yield with TPC, TFC, and DPPH radical scavenging ability	Higher temperature showed the decreased yield	Park *et al*. (2022)
Hog plum	Total phenolic compounds	52	The TPC, DPPH, and FRAP were 370 ± 26 mg GAE/100g DM, 57 ± 7%, and 7650 ± 460 mg AAE/100 g DM, respectively.	The decline in extraction yield can also be attributed to some minor degradation of unstable polyphenols at high temperatures and extraction time.	Ahmed *etal*. (2022)

2.4 Optimization of UAE using Response Surface Methodology

Generally, UAE is affected by several parameters including ultrasonic power, solvent concentration, pulse, amplitude, extraction time and temperature. In order to achieve optimum extraction yield, an optimization model of the most relevant operational parameters is required. Response surface methodology (RSM) is a mathematical and statistical technique that has been successfully applied investigating the possible interaction of experimental variables in various process (Gu *et al*., 2012).

RSM is a collection of statistical and mathematical techniques that are based on the fit ofempirical models to the experimental data. It is a useful tool in developing, improving and optimizing processes. RSM enables users to analyze the individual and interaction of independent variables on the performance of processes and generates mathematical models (Sanou *et al*., 2023). RSM has been introduced since 1950s and was further applied successfully to optimize various types of UAE systems (Sanou *et al*., 2023; Hundie & Ketema, 2020; Abdulgader *et al*., 2020; Xinsheng *et al*., 2013). This approach is becoming more popular because it offers larger amount of information from small number of experiments compared to the classical one-variable-at-time technique and provides analysis on the interaction effect of independent variables on the response (Sanou *et al.,* 2023).

There are generally four stages involved in optimization using RSM (Sanou *et al.,* 2023).The first stage is the selection of independent variables with major effects on the system.The second stage is the choice of experimental design

and carrying out the experiments according to the selected mathematical matrix. It is followed by selection of model as a function of independent variables and the statistical analysis and evaluation of the model. The last stage is obtaining the response surface plot and determination of optimum points. The literatures on the optimization of extraction process for various natural products using UAE system have been summarized in Table 2.11 below;

Table 2.11 : Optimization of extraction process for various natural products using Response Surface Methodology

Plants	Experimental Design	Optimized parameters	References
Roselle	Box-Behken Design	41.81 mg/mL; 52.35 °C and 57.77 min	Sanou *et al*. (2023)
Pomegranate peel	Central Composite Design	25 min, 59% methanol, 1:44 and 80 °C.	Zivkovic et *al.* (2018)
Red dates	Box-Behken Design	30ml/g, 70% ethanol, 60°C and 60 min	Bee *et al.* (2018)
Betel	Box-Behken Design	51.60 °C, 78.74% methanol, 1:21.85g/mL.	Ameena *et al.* (2018)
Lemon balm	Central Composite Design	33 min, 371W and 1.4% of ethanol	Caleja *et al.* (2017)
Licorice	Central Composite Design	69°C, 34 min, and 57% of ethanol	Jang *et al.* (2017)
Thymus vulgaris	Box-Behken Design	45°C, 3h and 20mL ethanol	Malik *et al.* (2016)
Jatropha	Central Composite Design	96%, 60°C, and 6h	Mohammad *et al.* (2021)
Black pepper	Central Composite Design	30 MPa, 40 °C and 40 min.	Bagher *et al.* (2014)
Mulberry	Central Composite Design	63.8% methanol, 43.2 °C, 23.8 (v/w) and 40min	Zou *et al.* (2011)

2.5 Extraction Kinetics

Kinetic study is essential to qualitatively measure the changes in chemical reaction suchas during solid-liquid extraction (Heldma, 2011). As a result, the research would be able to anticipate the extraction rate (the rate at which

equilibrium is reached), the mechanisminvolved in the reaction and provide an estimation of cost efficiency (time, cost, and energy) benefits to industrial applications (Galgano *et al.,* 2021; Da Porto and Natolino 2018).

The kinetic behaviour of solid-liquid extraction was explained in engineering terms by numerical solutions to Fick's second law of diffusion. The diffusion process was considered to involve with two mechanisms, which are internal and exterior diffusion (Chua, Poh, and Ameena 2018; Patrautani *et al.,* 2019). The mass transfer mechanism in the extraction process can be characterized using an extraction curve. The method typically comprises of two stages, which are rapid extraction and slow extraction steps (Chan *et al.,* 2014). The rapid extraction process occurs when the solute is initially exposed to the solvent. The process may involve the penetration and swelling of solvent into the outer plant surface, followed by the dissolution of soluble solutes into a solvent (Goula 2013; Mitic *et al.,* 2021). This stage permits the rapid diffusion of solute due to thebreakage of the cell wall.

2.6 Extraction Kinetics Models

The most significant element of kinetic modelling is evaluating the mass transfer mechanism. The mechanism is important to predict the effect of process variables that changed throughout the process (Setford *et al.,* 2019). Most of the extraction kinetic models account for the intensity of process parameters such as temperature, frequency,pressure, particle size and solid-to-liquid ratio. These parameters affect the recovery of specific components as a function of time (Patrautani *et al.,* 2019; Lazar *et al.,* 2016; Hadidi, Ibarz

and Pagan, 2020). Thus, it is necessary to develop kinetic models based on several process parameters.

Numerous mathematical models were developed based on Fick's second law of diffusion to have in-depth kinetic exploration in solid-liquid extraction (Chua *et al.,* 2018; Patrautani et al., 2019). According to Hobbi *et al.* (2021), the second-order kinetic model was sufficient to describe the extraction mechanism of total phenolic compounds from apple pomace using UAE with the highest TPC yield of 11.1 ±0.49 mg gallic acid equivalent (GAE)/g db (dry basis) with a mixture of 65% acetone–35% water (v/v) at 60 °C. Balyan *et al.,* 2016 claimed that the first-order model seemed to be more appropriate among other models to represent the extraction of total polyphenols from jamun seeds in extraction of tannic acid, gallic acid, ellagic acid and caffeic acid which was about 82% recovery using UAE. Table 2.12 presents the previous research on extraction kinetics using different models and various plant materials for extraction of similar product of interest.

Table 2.12 : Extraction kinetics using different kinetic models and plant materials

Models	Bioactive compounds and Plants	Extraction Methods	Optimal Kinetic Parameters	Conclusion	References
First order	C-phycocyanin from *S. platensis*	UAE	30 min, 45°C, 100 kHz of ultrasonic frequency, and solid-liquid ratio of 1:5 w/v.	The study showed efficiency of the UAET, as an efficient method to produce the CPC extracts	Phirunrat *et al.* (2019)
Second order	*o*-coumaric acid from Black Quinoa	UAE	10 min, 20 °C and 1:20 g mL^{-1} of solid to solvent ratio	Optimizing the UAE provides great extraction of phenolic compounds and best modelled by second order rate	Melini *et al.* (2021)
	Flavonoids and triterpenes from Chinese northeast black bee propolis	UAE	80% ethanol concentration, 30:1 mL/g ratio, 53 °C, 16 min and 10s/5s on-time/off-time.	UAE showed higher extraction yield compared to conventional extraction (48.55%), which was achieved in shorter time	Qingzhi *et al.* (2018)
Peleg	Agrimoniin from strawberry peels	UAE	1:30 extraction ratio, 40% ethanol concentration, and 100% ultrasound power	Kinetics extraction of agrimoniin was successfully demonstrated by Peleg's Model	Villamil-Galindo *et al.* (2023)
	caffeic, coumaric,trans-cinnamic, 4-hydroxybenzoic, and syringic acids from *Psidium cattleianum* leaves	UAE	60% sonication amplitude at 0.6 s of the pulse cycle for 4 minutes	UAE improves the yield extraction of the PC leaf phytochemicals better than conventional	Gonzalez-Silva *et al.* (2022)

2.6.1 First-Rate Order

The first-rate order kinetic model is the most straight forward equation. It was developed according to Fick's Law of diffusion (Chua, Poh, and Ameena 2018; Poojary and Passamonti 2015). The Equation 5.2 can be written as follows.

$$\frac{dC_t}{dt} = \kappa_L A[C_s - C_t] \tag{2.10}$$

where dC_t/dt represents the rate of solute, k_L is denoted as mass transfer coefficient, A is the total surface area of solid, C_s is the extraction capacity (concentration of solute at saturation in g/L) and C_t is the concentration of solute in the solution at any time t (min). After integrating the equation with boundary condition $C_t = 0$, $t = 0$, the final equation is expressed in Equation 2.11.

$$C_t = C_s\,[1 - e\,(-k.\,t)] \tag{2.11}$$

According to (Poojary and Passamonti 2015), the plot of C_t versus against time that describes the extraction kinetics typically shows an exponential decay. Equation 5.3 can be converted into a linear form as below;

$$\log(C_s - C_t) = \log\,(C_s) - \frac{k}{2.303}t \tag{2.12}$$

The linear plot of log ($Cs - Ct$) versus t could determine the first-order extract rate (k) andextraction capacity given by a slope and intercept, respectively (Kusuma and Mahfud 2017; Alara and Abdurahman 2019).

2.6.2 Second-Rate Order

A second order process typically takes place in two subsequent stages. The first stage isthe rapid extraction of the major parts of the solutes due to the scrubbing and dissolution caused by the driving force of the solvent. The second stage is the slower extraction process due to external diffusion of the remainder solute into the solution (Lau *et al.,* 2015; Kaderides *et al.,* 2019). The second order kinetic rate equation which represent the dependence of the extraction rate on the solute dissolved in the solution is expressed as follow;

$$\frac{dC_t}{dt} = k\left(C_s - C_t\right)^2 \tag{2.13}$$

Where k is the second order extraction rate constant (L/mg min), C_s is the extraction capacity (concentration of solute at saturation in mg/L) and C_t is the concentration of solute in the solution at any time *t* (min).

By considering the boundary condition t=0 to t and C_t=0 to C_t, the integrated rate law for a second-order extraction is obtained as follow (Lazar et *al.,* 2016);

$$C_t = \frac{C_s{}^2 kt}{1 + C_s kt} \tag{2.14}$$

The linearized above equation would be;

$$\frac{t}{C_t} = \frac{1}{kC_s{}^2} + \frac{t}{C_s} \tag{2.15}$$

The extraction rate can be further written as;

$$\frac{C_t}{t} = \frac{1}{\left(\frac{1}{kC_s^{\,2}}\right) + \left(\frac{t}{C_s}\right)} \tag{2.16}$$

Then, as *t* approaches 0, the initial extraction rate, *h* as C_t/t can be expressed as;

$$h = kC_s^{\,2} \tag{2.17}$$

2.6.3 Peleg

The equation describes the extraction curve of the medicinal plants which followed theshape of the sorption curve (Chua, Poh and Ameena 2018). The equation is written as below;

$$C_t = C_o + \frac{t}{k_1 + k_2\, t} \tag{2.19}$$

C_o represents the initial solute concentration at t, C_t represents the solute concentration at t, k_1 is the Peleg's rate constant, and k_2 is the Peleg's capacity constant. When the initial concentration is zero, C_o can be omitted. The final equation is written as below;

$$C_t = \frac{t}{k_1 + k_2 t} \tag{2.20}$$

2.7 Bioconversion of Dicoumarol from O-Coumaric Acid

2.7.1 Submerged Culture System

According to Vezina *et al.* (2015), submerged culture is referred to as the

technique of growing an organism in the liquid medium that was maintained under aerobic conditionsby agitation of the culture vessel (shake flask system) or by introduction of air into agitatedmedia in stationary container (bioreactor system). Agitation is one of the most important parameters in aerobic submerged fermentation, since it ensures homogeneity with respect to temperature and gaseous environment and provides gas-liquid interfacial area for gas to liquid as well as liquid to gas transfers.

Agitation also promotes surface mass, heat transfers and uniform distribution of nutrientsadded incrementally during the course of fermentation. Growth of aerobic microorganisms in submerged culture is controlled by the availability of substrates, energy and enzymes. Cultures are always of a heterogeneous nature and so rates of reactions can be limited by the rate of substrate or product transfer at a particular interface.

2.7.2 *Aspergillus Fumigatus Fresenius* NRRL 163

The genus *Aspergillus* is one of the most important filamentous fungal genera and most commonly used in fermentation industry (Perrone *et al.,* 2017). *Aspergillus fumigatus Fresenius* NRRL 163 was chosen for this study as suggested by Bye and King (1970) who had reported that strain has been characterized as a producer of dicoumarol which have been responsible for the key events involved in the biosynthesis of 4- hydroxycoumarin and dicoumarol. The strain isolated from spoiled hay of *Melilotus alba*, converted melilotic acid (o-hydroxyphenylpropionic acid) and O-C into 4- hydroxycoumarin and dicoumarol.

Bellis *et al.* (1967) has also worked on biosynthesis of dicoumarol using *Penicillium jenseni* isolated from *Melilotus officinalis*. During the metabolism of O-C by the strain, 4-hydroxycoumarin was identified in the culture medium long before the appearance of dicoumarol. 4-Hydroxycoumarin was further converted into dicoumarol by treatment with formaldehyde at room temperature.

A. fumigatus NRRL 163 is commonly regarded as a single homogenous species but thestrains may differ depending on their cultural activities and to a lesser degree in their morphological characteristics. *A. fumigatus* NRRL 163 isolates has produced dark-blue greenish colonies in potato dextrose agar and basal salt agar plates, consisting of a dense of conidiophores intermingled with aerial hyphae.

Literaturely under microscopic observation as in Figure 2.9, the conidial heads were columnar and quite densely compact. The conidiophores were smooth, short up to almost300µm in length, commonly more or less to green in colour and arise directly from submerged hyphae or as obviously short branches from the aerial hyphae. The vesicleswere subclavate up to 20 to 30µm in diameter and usually similar in colour to the conidiophores. Phialides were directly borne on the vesicle, pigmented and were roughlyabout up to 8µm long. The conidia were mostly green in colour, smooth to finely echinulate, globose to subglobose and mostly 2.5 to 3.0µm in diameter.

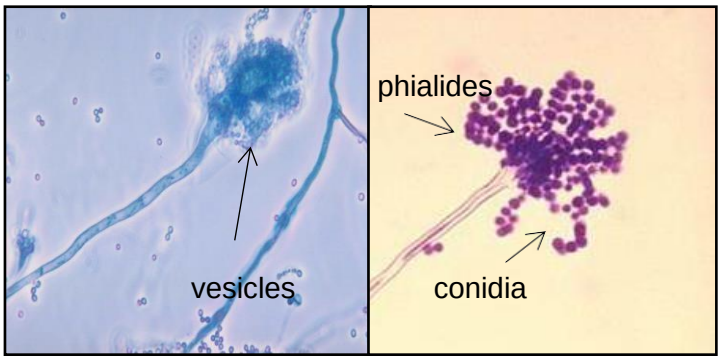


Figure 2.9 : Conidial morphology of *A. fumigatus Fresenius* NRRL 163
(Source: https://www.wikiwand.com)

2.7.3 Development of Fermentation System

Microbial growth is greatly affected by chemical and physical nature of their surroundings. For successful cultivation of microorganisms, it is not only essential to supply proper andbalanced nutrients but also it is necessary to maintain proper environmental conditions. Growth and death rates of microorganisms are greatly influenced by nutrient supply. Carbon sources supplied in the medium are of great importance to any microorganism since they provide the carbon needed for the biosynthesis of cellular constituents such as carbohydrates, proteins, lipids, nucleic acids and their oxidation provides energy for the cell (Khani *et al.,* 2016). Normally, glucose is fed as a sole carbon source in order toachieve high cell density culture of *A. fumigatus* NRRL 163.

Most industrial strains are capable to utilize inorganic or organic nitrogen sources. Inorganic nitrogen may be supplied by ammonia gas, ammonium salts or nitrates while organic sources may be supplied as amino acid, protein, peptone, yeast extract or urea Complex organic nitrogen sources such as

peptone and yeast extract may contain vitamins, which acts as a precursor for dicoumarol production (Kaur *et al.,* 2021). Furthermore, some organic nitrogen sources have a good buffering system while inorganic nitrogen sources such as ammonia, reduces the culture pH excessively during the NH_4^+ adsorption.

Das *et al.* (2012) reported on their study using response surface methodology to optimize mixed substrate solid state fermentation for cellulases and xylanase production by *Aspergillus fumigatus* ABK9. Among 11 different parameters, incubation time (86–88 h), medium pH (6.1–6.2), mass of substrate (10.0–10.5 g) and substrate ratio (wheat bran: rice straw) (1.1) had significantly influenced on the enzyme production. Under these conditions, endoglucanase, b-glucosidase, FPase (filter paper degrading activity) and xylanase activities of 826.2, 255.16, 102.5 and 1130.4 U/g, respectively were obtained.

Rohit *et al.* (2021) reported the optimization of cellulase production by *Aspergillus fumigatus fresenius* for enzymatic hydrolysis of Solka floc and bagasse were successfully produced maximal levels of cellulase on basal salt medium containing rice straw as carbon and beef extract as nitrogen source at 45 °C. They added that the optimization of cellulase production which was carried out using Box–Behnken design of experiment resulted concentrated culture extracts which efficiently hydrolyzed Solka floc and bagasse (7% w/v) resulting in 90 and 87% saccharification, respectively. Praveen *et al.* (2019) indicated that initial dye concentration of 150 mg/L, pH 5.5 and a temperature

of 30 °C were optimal for maximum % decolorization of Acid Red 151 (AR 151) in simulated dye solution using a fungal isolate *Aspergillus fumigatus Fresenius*. They claimed that decolorization was observed at optimum growth conditions with 84.8% decolorization of AR 151.

2.7.4 Mechanism of Bioconversion of Dicoumarol

Bye and King (1970) had reported their work on the biosynthesis of 4-hydroxycoumarin and dicoumarol by *Aspergillus fumigatus Fresenius* using *Mellilotus alba* (sweet clover). They stated that mellilotic acid (o-hydroxy-phenylpropionic acid) and O-C can serve as precursors for dicoumarol production. The mechanism of the biosynthesis of dicoumarol from O-C is initialized by the esterification of O-C by xanthate reaction as stated in Figure 2.10.

Initially, O-C induces its own conversion into 4-hydroxycoumarin. The substantial amounts of -hydroxymelilotic acid and –oxomelilotic acid escape into the medium suggests that the metabolites do not remain firmly bound to an enzyme surface throughout the sequence. The reaction continues with semicarbazide in the presence ofO-C, with dicoumarol as an end product

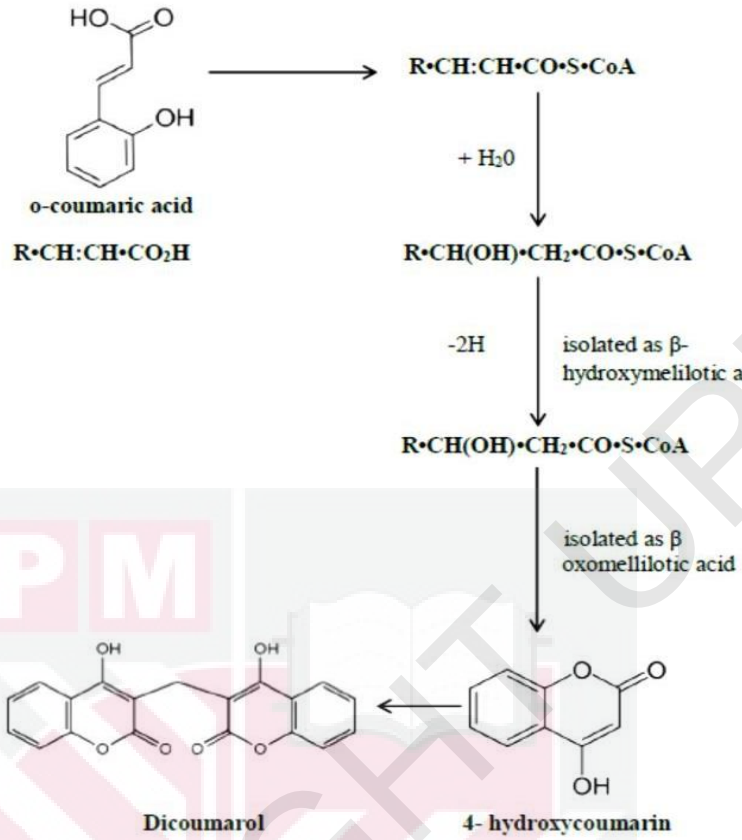


Figure 2.10 : The dicoumarol formation from melilotic acid and *o*-coumaric acid through several reaction sequences
(Source: Bye and King, 1970)

2.7.5 Microbial Growth Kinetics in Batch Culture

The kinetics of cell growth is substantially capable to predict the formation of product fromfermentation process. The kinetics describes growth and product formation not only during active cell growth but also during resting and dying cells activities. Kinetics are concerned with reaction rates in general in which kinetic process simply suggest a primary concern with the rate of commercially practiced reaction and particularly, with theeffects of process variable on them (Gaden & Elmer, 2000).

The kinetics of microbial growth, substrate uptakes and product fermentation process can be described using mathematical models Models are used to describe microorganism's behaviour under different physical and chemical conditions such as temperature, pH andwater activity and also can be used in the process control and optimization (Sakthiselvan*et al.,* 2020). Kinetic growth is related as a crucial step in developing a fermentation process since the kinetic study can be used to analyse an optimal operation condition forthe production of a target metabolite (Du *et al.,* 2022).

Microbial growth kinetics explains the relationship between the specific growth rate of a microbe and its substrate concentration. In batch culture, microbial cell composition andits state change as a function of time and thus the rate of increase in biomass concentration is monitored (Sakthiselvan *et al.,* 2020). Shake flask fermentation is one ofthe examples of batch fermentation. Batch culture is an example of a closed culture system which contains an initial, limited amount of nutrient as shown in Figure 2.1.

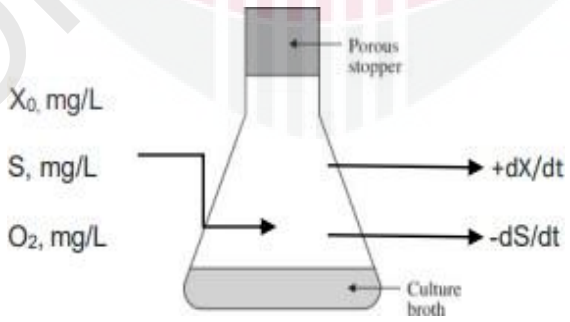


Figure 2.11 : Schematic diagram of microbial growth and substrate utilization in a well-mixed shake flask

The inoculated culture will pass through a number of phases. After an inoculation there is a period during which no growth appears to take place. The flask initially contains a known growth substrate concentration, S. The flask is well mixed and therefore the dissolved oxygen concentration, O_2 does not become a limiting factor for microbial growth. Initially a known concentration, X of viable microbial cells is inoculated into the container and substrate S is utilized with time for the cell growth. Then, an observation ofa decrease in substrate and a corresponding increase in biomass. A conceptual plot of microbial cell concentration vs time for the batch system is calleda growth curve, as shown in Figure 2.12.

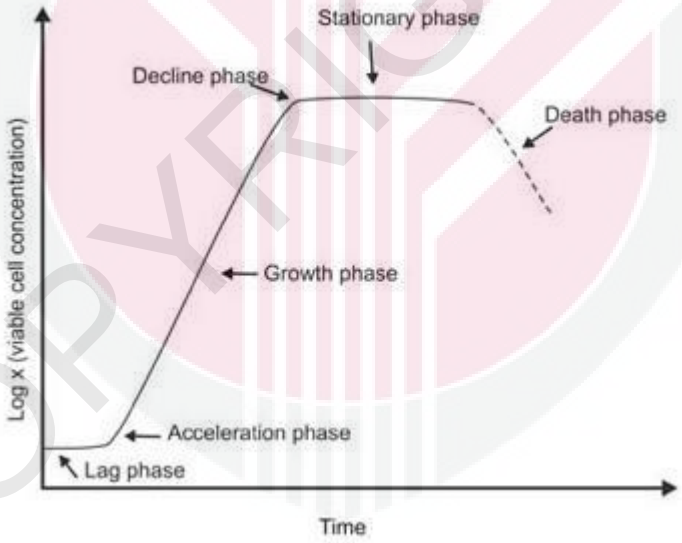


Figure 2.12 : Typical growth curve for a batch system

By plotting the log of viable cell concentration, X, with time, five distinct phases of the growth curve can be identified; 1) the lag phase which occurs immediately after inoculation and persists until the cells acclimitisation to their new environment, 2) exponential growth phase, during which time cell

growth proceeds at an exponential rate, 3) a deceleration phase, when essential nutrients are depleted and secondary products begin to accumulate, 4) a stationary phase during which time the net cell growthis approximately zero, and 5) death phase where some cells loose viability or are destroyed by cell lysis.

During the lag phase, dX/dt and dS/dt are essentially zero. However as exponential growth phase begins it is possible to measure dX/dt and dS/dt values which are very useful for defining important microbial kinetic parameters. Using corresponding observations of dS/dt and dX/dt obtained just after the onset of exponential growth phase,the yield coefficient, $Y_{X/S}$ and the specific growth rate μ are computed as;

Yield coefficient, Y (Liu and Yu, 2017);

$$Y = \frac{dX}{dS} \tag{2.21}$$

Where;

dX is mass of new cells

dS is mass of substrate consumed

Specific growth rate, μ (Liu and Yu, 2017);

$$\mu = \frac{\mu_{max}\,[S]}{k_s + [S]} \tag{2.22}$$

Where;

μ_{max} is maximum specific growth rate

k_{max} is monod saturation constant

S is subsrate concentration, g/L

Equation 2.22 is further simplified as;

$$\frac{1}{\mu} = \frac{k_s}{\mu_{max[S]}} + \frac{1[S]}{\mu_{max}\,[S]} \tag{2.23}$$

Multiplying both side equation with S then;

$$\frac{[S]}{\mu} = \frac{k_s}{\mu_{max}} + \frac{1}{\mu_{max}} \tag{2.24}$$

The yield coefficient, commonly referred to as the substrate-to-biomass yield, is used to convert between cell growth rate dX/dt and substrate utilization rate dS/dt. The yield coefficient and the specific growth rate are used to develop microbial growth kinetic relationships.

CHAPTER 3

RESEARCH METHODOLOGIES

3.1 Introduction

The research approach focuses on the three main processes including oven drying of *Gliricidia sepium* leaves, leaves extraction and bioconversion of bioactive compound. The overall process was illustrated in Figure 3.1;

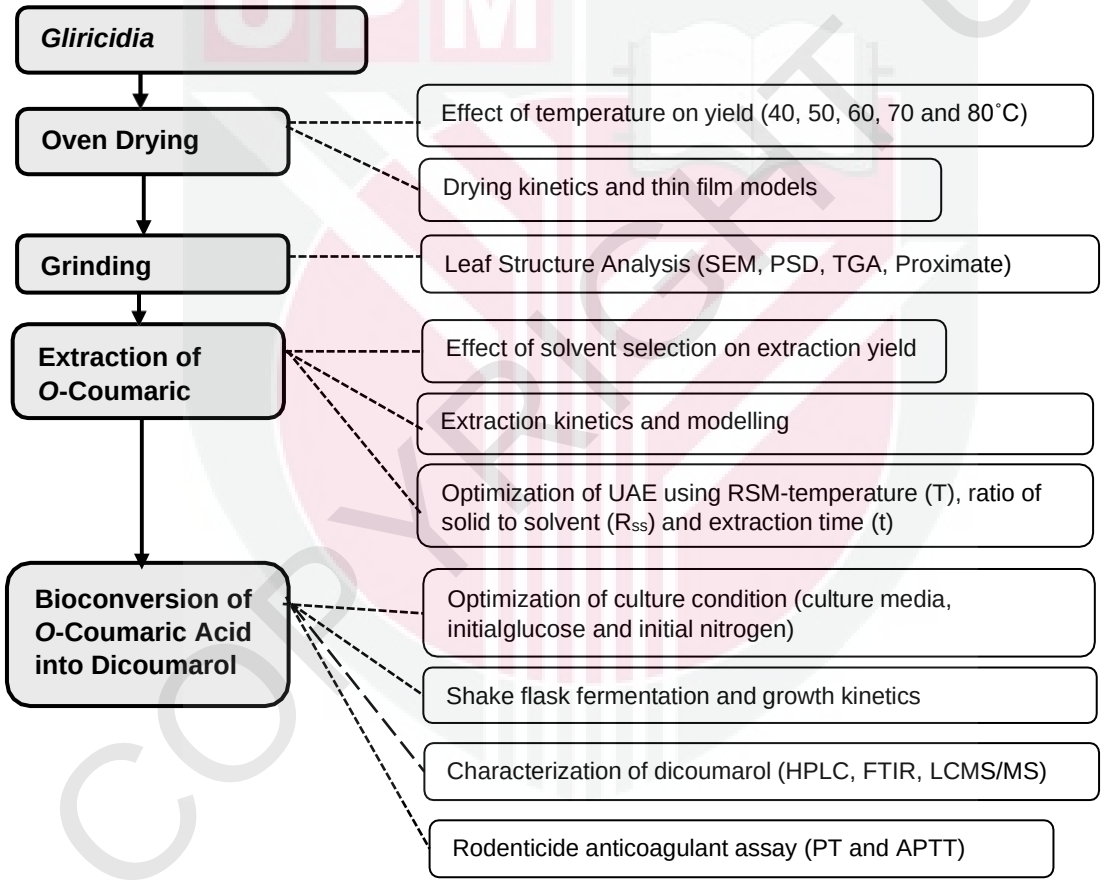


Figure 3.1 : Overview of the overall process flow

Based on Figure 3.1, in overall, the leaves of G. *sepium was* initially collected, preserved in wax-quoted paper bags and brought to the laboratory. After sample verification, the leaves were let to oven drying under several

temperatures in range o 40-80˚C. The drying process was studied on its kinetics and thin layer modelling. The dried leaves were thenlet to grinding process with specific particle size distribution and moisture content as pre-requisite of the optimal extraction process. The grounded leaves were also let to be captured under scanning electron micrography for pore size observation.

On the second stage, the extraction process was studied in term of several affecting parameters including the extraction method (SE and UAE), effect of solvent selection (types and concentration), extraction time, temperature and ratio of solvent to solid on quality and quantity of the extraction yield. The extraction kinetics and modellling were further studied. The best selected extraction method was then optimized using ResponseSurface Methodology using central composite design.

On the last part, the bioncoversion process was started with the optimization of batch shake flask fermentation process using several parameters (culture media, initial glucoseand initial nitrogen). The growth kinetics were studied to determine the optimum conditions of fermentation process. The extracted *o*-coumaric acid and converted (dicoumarol) compound were confirmed through HPLC, FTIR and HPLC-LMS/MS. The research was ended with the anticoagulant screening test using Prothrombin Time and Activated Partial Thromboplastin Time methods.

3.2 Raw Materials

3.2.1 Chemicals and Reagents

Sodium Nitrate ($NaNO_3$), Potassium Chloride (KCL), Magnesium Sulphate Heptahydrate($MgSO_4$. $7H_2O$), Potassium Dihydrogen Phosphate (KH_2PO_4), Iron (II) Sulphate Heptahydrate ($FeSO_4$. $7H_2O$), Zinc Sulphate Heptahydrate ($ZnSO_4$. $7H_2$) and D-Glucosewere purchased from Sigma Aldrich, Inc., USA. The strain of *A. fumigatus* NRRL 163 was purchased from American Type Culture Collection, United States (ATCC 1022D ^{TM}) whilethe internal standard of O-C and dicoumarol were purchased from Sigma Aldrich, Inc., USA. Potato Dextrose Agar (CM0139) and Potato Dextrose Broth (CM0139B) were purchased from Oxoid, UK. All other reagents used in the study were of analytical grade.

3.2.2 Plant Material Authentication and Registration

The leaves of *G. sepium* were collected along some local residential areas in Nilai, NegeriSembilan, preserved in wax-quoted paper bags and brought to the laboratory for storage. The samples were deposited in Biodiversity Unit, Institute Bioscience (IBS), Universiti Putra Malaysia for species identification and verification. The voucher specimen was authenticated and registered by Mohd Hafizi Adzmi Hanafi with registration number SK 3133/17. The collected leaves were tested chemically using High Performance Liquid Chromatography prior to further processes for quality consistency test and pre-screening purposes.

3.2.3 Materials Characterisation

The dried leaves were initially ground into powder form using a grinder (Restech, ModelSM-100, Germany). The powdered leaves were sieved using laboratory mesh siever (Fisher Scientific, Model Cole-Parmer 35 mesh, US). The specific surface area of the powdered leaves was determined by using Particle Size Analyzer (Malvern, Model Mastersizer 2000, UK). The analyser uses blue LED light with 446nm of beam wavelength. The leaves morphology was observed using Scanning Electron Microscopy(JEOL, Model JED-2300, Germany). The thermal behaviour of *G. sepium* had been studied by Thermo Gravimetric-Differential Thermal Analysis (Hitachi, Model STA 200, China).

3.2.4 Strain Collection

The fungal, *A. fumigatus* NRRL 163 was used in this study and it was proposed by the literature to be the best cell lines in bioconversion of dicoumarol (Bellis *et al.*, 1967). Thestrain was directly purchased from American Type Culture Collection (ATCC) in ampoule containing viable cell lines suspended in cryoprotectant. The stock culture was freeze dried and the working cultures were maintained by weekly subculturing on potato dextrose agar and stored at 4-8°C.

3.2.5 Animal Blood Collection

The blood from rat (*Male Sprague Dawley*) was chosen for anticoagulant test because the species was widely used in previous related study by Manjusha *et al.* (2014) while the mean mass body weight was selected based on the

study conducted by Nowland *et al.* (2011). Five male adult white rats of Sprague Dawley at age of 1-2 months were collected from Animal Resource Unit (ARU), Department of Veterinary Pathology and Microbiology, Universiti Putra Malaysia. The blood sample was obtained from Pet Clinic and Surgery, Nilai, Negeri Sembilan. All the blood collection works were attended by a certified veterinarian with animal ethic permission (practicing certificate number AM18- 000035-OC). The rats were brought to the lab in specific cage and their respective bodyweights were recorded.

3.3 Oven Drying of *Gliricidia sepium* Leaves

3.3.1 Effect of Drying Temperature on Extraction Yield

The drying experiment was carried out by using an oven dryer (Memmert, Model UFP- 800, Germany). The oven dryer is equipped with temperature and speed controller of suction fan. The speed of suction fan was set at maximum throughout the experiment. The leaves were dehydrated under five different temperatures; 40, 50, 60, 70 and 80°C. The final reading of drying process was recorded when constant weight was observed.

The sample weight loss was measured at an interval of 10 minutes using electronic balance (Sartorious, Model PW214, Germany). The temperature of ambient air was between 26 to 28°C while the relative humidity of air was between 80 to 88%. The initial moisture content of fresh leaves was recorded as 81.37% on wet basis using Halogen Moisture Analyzer (Mettler Toledo, Model HB43, USA). For the determination of chemical profile and concentration of *o*-coumaric acid, the High Performance Liquid Chromatography (Model

Shimadzu, SPD 20A) method was used. The fine grounded leaves of 1g were mixed with 30ml of water and lysed for 20 minutes at room temperature using sonicator (Model 2510, QSonica, USA) for the rupture of plant cells. The extraction of phytochemicals was enhanced through UAE technique followed by filteration using syringe filter (diameter: 17mm, porosity: 0.45µm, PVDF membrane, TITAN, USA) for filtration of solid particles. The 10.0µL filtrate was injected at into HPLCfor further analysis.

3.3.2 Effect of Drying Temperature on Drying Kinetics

The drying process was carried out using oven dryer with five temperatures at 40, 50, 60,70 and 80°C. The range was selected based on single experiment that has been closely done using 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100°C. The selected five temperature ranges of 40-80°C have enough shown the significant effect on the extraction yield. The fresh *G. sepium* leaf of 10.0g was placed in a perforated aluminiumtray (40cm x 30cm) as shown in Figure 3.2.

The interval time for weight loss measurement was selected as 5 minutes for 80 and 70°C, 10 minutes for 60 and 50°C. The weight loss for the dried sample at 40°C was measured at 10 minutes' interval for 3 hours, 20 minutes for the following 4 hours and 30minutes until constant weight was achieved. This is because the drying rate for 40°C is slow but the total drying time required is relatively long. The experiment was stopped once there was no weight loss observation after thrconsecutive measurements. All the experiments were conducted in triplicates.

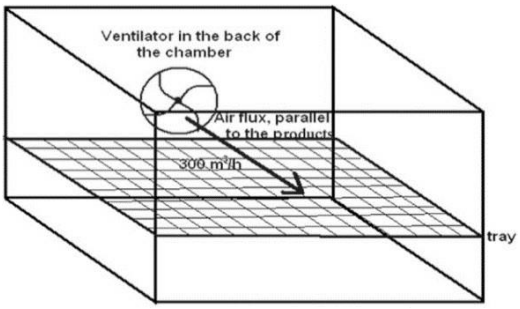


Figure 3.2 : A schematic diagram of drying chamber
(Source: Guine *et al.,* 2002)

3.3.3 Mathematical Modelling for Drying Kinetics

Process modelling is an important element for the dryers' design and operation at optimaldrying constants. Drying kinetics using thin-layer kinetic modelling has been applied to many biological materials such as tea (Anindita & Partha, 2018), mate (Shinde *et al.*, 2013) and kaffir lime (Trirattanapikul *et al.*, 2014) for predicting which model best describes the drying process.

Five thin layer models were evaluated to find the most suitable model to describe the drying process as listed in Table 3.1. These models were selected as they were found applicable on the drying of various materials as discussed in Chapter 2. It is assumed that the drying process is controlled by the external resistance between the solid materials and drying air. The reduction of the moisture ratio with drying time of *G. sepium*leaves was used to analyse the experimental data. The moisture ratio represents the amount of moisture remained within the *G. sepium* leaves from the initial moisture content. The dimensionless moisture ratio is given by Equation 3.1 (Nguyen *et al.,* 2019);

$$MR = \frac{M - M_e}{M_o - M_e} \tag{3.1}$$

Where *M* is the moisture content at any time of the drying, M_o is the initial moisture content and M_e is the equilibrium moisture content at which the material is neither gaining nor losing moisture. Correlation coefficient (R^2) and root meant square error (RMSE) were the criteria selected to evaluate the agreement between the experimental data and the predicted models. These criteria were calculated Equations 3.2 and 3.3 (Nguyen *et al.*, 2019) respectively.

$$R^2 = \left[\frac{\sum_{i=1}^{N} \left(MR_i - MR_{pre,i}\right)^2 * \left(MR_i - MR_{\exp i}\right)}{\sqrt{\left[\sum_{i=1}^{N} \left(MR_i - MR_{pre,i}\right)^2\right] * \left[\sum_{i=1}^{N} \left(MR_i - MR_{\exp i}\right)^2\right]}} \right] \quad (3.2)$$

$$RMSE = \left[\frac{1}{N} \sum_{i=1}^{N} \left(MR_{pre,i} - MR_{\exp i}\right)^2 \right]^{1/2} \quad (3.3)$$

R^2 is the coefficient of determinant, $MR_{exp,}$ is the experiment moisture ratio in any measurement, $MR_{pre,\ i}$ is the predicted moisture ratio for the measurement and N is the total number of observations. The R^2 value nearest to 1 was the best choice as it is the primary criteria for selection of model. The value of R^2 is close to unity and the value of RSME is close to zero represents a better agreement between experimental results andpredicted data.

Many models have been applied to describe the drying process for different agricultural products. The thin layer models are used to estimate drying time of several products under different drying condition, to enhance the drying process efficiency and also to generalize the drying curves. Table 3.1 shows five thin layer models which commonly used in mathematical modelling for

drying time of leafy plant materials.

Table 3.1 : Five thin layer models used in mathematical modelling

Model Name	Model Equation	Reference
Page	$MR = exp(-kt^n)$	Mbegbu *et al.,* (2021), Karakaplan *et al.,* (2019); Panchariya *et al.,* (2002); Inyang *et al.,* (2018); Nurafifah *et al.,* (2018); Fall Beye *et al.,* (2017).
Modified Page	$MR = exp(-kt)^n$	
Lewis	$MR = exp(-kt)$	
Logarithmic	$MR = a\ exp(-kt) + b$	
Midilli *et al.*	$MR = a\ exp(-kt^n) + bt$	

Note: *t* is drying time while *a*, *b*, *k*, and *n* are defined as drying constant

3.3.4 Effective Moisture Diffusivity

The effective moisture diffusivities, D_{eff} are typically determined by plotting the experimental data of ln (MR) versus time. So, Fick's second law is used to describe the drying material of any materials in the falling rate drying period. The equation of moisture ratio was determined using Equation 3.4 (Alara *et al.,* 2019).

$$MR = \frac{M}{M_0} = \frac{8}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{(2n-1)^2}\exp\left[-\frac{(2n-1)^2\pi^2 D_{eff}}{4L^2}t\right] \quad (3.4)$$

Where L is the thickness of the slab and n is the positive integer. Then, Equation 3.4 was simplified to Equation 3.5 as below for longer drying tim

$$MR = \frac{M - M_e}{M_0 - M_e} = \frac{8}{\pi^2}\exp\left(-\frac{\pi^2 D_{eff}}{4L^2}t\right) \quad (3.5)$$

Equation 3.5 was further simplified to straight line as Equation 3.6 below

$$\ln MR = \ln\frac{8}{\pi^2} - \frac{\pi^2 D_{eff}}{4L_2}t \quad (3.6)$$

3.4 Quantitative Effects of Solvent System on Extraction Yield Using Soxhlet and Ultrasonic Assisted Extraction Extraction Methods

3.4.1 Effect of Solvents Types on Extraction Yield

There were five types of solvents used in the experiments including hexane (Hex), ethylacetate (EA), chloroform (Cloro), methanol (MetOH) and pure water (H_2O). The solvents were selected based on their polarity ranges from most non-polar, less non- polar, less polar, medium polar to the most polar. The polarity in increasing order was Hex > EA > Chloro > MetOH > H_2O.

The solvents used were of analytical grades. The extraction was carried out by using Soxhlet apparatus (Bionics, Model BST/SX-1A, India) and also ultrasound equipment (QSonica, Model Q500, New York) for yield comparison. The temperature of the extraction process was thoroughly monitored with thermometer. A low temperature of 50°C was selected as working temperature to elude degradation of phytochemicals as being suggested by previous works (Alara *et al*., 2019; Wu *et al.,* 2008).

The ratio of solvent to solid (R_{ss}) used in the extraction was 30:1 (ml: g). Process duration was selected as 30 minutes which was estimated to be sufficient for achieving equilibrium by referring to the most literatures in Chapter 2. After the extraction, the water extract was filtered using the vacuum filtration set. The filtration set is composed of funnel, collection flask and suction pump. The suction pump was used to accelerate the filtration process. The extracts from the rest solvents of Chloro, MetOH, EA and Hex were recovered by removing the solvents using rotary evaporator under

vacuum.

The recovery method was selected as the boiling points of these solvents were typicallylow. Furthermore, these solvents were not able to be frozen and thus freeze drying is notapplicable. The evaporation process was conducted at a temperature of 50ºC to minimize any possible of samples degradation. The extraction yield was defined as the percentage of the ratio of dried extract recovered to raw materials used which was given as:

$$Yield\,(wt\%) = \frac{W_d}{W_s} \times 100 \tag{3.7}$$

In the above equation, W_d and W_s are weight of dried extract and solid raw materials (g),respectively. All the experiments were conducted in triplicates.

3.4.2 Effect of Solvent Concentration on Extraction Yield

The experiment was designed to investigate the effect of solvent concentration on the extraction yield. The experimental conditions were listed in Table 3.2. The experiments were conducted in triplicates. The percentage of final solution was selected from range 10-90% (v/v) to closely examine the optimum volume of methanol solution.

Table 3.2 : Experimental setup for the effect of solvent concentration for Soxhlet and Ultrasonic Assisted Extraction Methods

No.	Volume of methanol (ml)	Volume of water (ml)	Percentage of Solution (% v/v)
1.	10	90	10
2.	20	80	20
3.	30	70	30
4.	40	60	40
5.	50	50	50
6.	60	40	60
7.	70	30	70
8.	80	20	80
9.	90	10	90

The dried extracts (1.0 mg) from Chloro, MetOH, EA and Hex were dissolved in 1.0 ml of methanol. The solutions were then filtered with syringe filter (diameter of 17mm, porosityof 0.45µm, PVDF membrane, TITAN, USA) before HPLC analysis. The injection volume of the filtrate was 10.0□l. All the experiments were conducted in triplicates.

3.5 Extraction Techniques for Bioactive Compound Extraction

3.5.1 Soxhlet Extraction

Five grams of *G. sepium* leaves sample was put into extraction thimble and the thimble was transferred into a Soxhlet apparatus (Bionics, Model BST/SX-1A, India).Approximately, 150 mL of 80% methanol were added to each flask which was connected to the extractor. Each extraction was performed in triplicate for 6 hours. The extracts were weighed gravimetrically.

3.5.2 Ultrasonic Assisted Extraction

Five grams (5 g) of *G. sepium* leaves sample were extracted in a beaker (100 ml) containing a volume of extraction solvent. The extraction solvent was methanol (80%) which was obtained from the previous works. The extraction was performed by ultrasonicequipment (QSonica, Model Q500, New York) at constant power of 130 W and frequencyof 20 kHz.

Temperature was monitored by immersing the beaker into an automatically temperature control water-bath (Memmert, Model WNE14, Germany) and no significant increase in temperature (below 2ºC) was observed during the extraction process. Control experiments were carried out without using ultrasonic waves. The immersed leaves in extraction solvents were subjected to ultrasonic waves during a period of 20 to60 min. After extraction, the crude extracts were filtered through the filter paper.

Then, methanol was removed from the extracts by evaporation under vacuum at 40ºC using a rotary evaporator. Subsequently, the residual solvent was removed by oven drying at 40ºC for 1 h and by flushing with 99.9% nitrogen. Gravimetric measurement was used toobtain the amount of total crude extract yield (CEY) using Equation 3.8 (Alara *et al.,* 2019).

$$CEY = \frac{m_e}{m_s} x1000 \tag{3.8}$$

The results of CEY were expressed as mgg^{-1} sample, where m_e is the crude extract mass (g) and m_s is the extracted sample mass (g). The extract

samples were kept for furtheranalysis.

3.6 Identification of *O*-Coumaric Acid from Crude Extract

3.6.1 Identification of *O*-Coumaric Acid Using High Performance Liquid Chromatography

A commercial grade of *o*-coumaric acid purchased from Sigma-Aldrich (USA) was selected as qualityindicator in this work. It is because the compound was reported to be mainly found in *G.sepium* leaves other than coumarin and it contributes to the bioconversion work as beingdiscussed in Section 2.1.3.

A ZORBAX Eclipse Plus C18 column (250mm x 4.6mm, 5 µm particle size, USA) was used as stationary phase. The column was rinsed for each of 10 column volumes with water: acetonitriles (95:5 v/v). The mobile phase was in gradient mode and consists of methanol and 0.4% acetic acid (80:20 v/v). The combination of mobile phase was selected for better separation. The detection wavelength was 290nm as the compound is sensitive to the wavelength. The experimentswere conducted in triplicate for the better reliability. The changes of mobile phase contentduring the analysis were shown in Table 3.3.

Table 3.3 : Mobile phase changes in HPLC analysis for *O*-Coumaric Acid

Time (min)	0.4% Acetic Acid, C2H4O2 (20v/v)	100% Methanol, MeOH (80v/v)
5	80	20
10	40	60
15	20	80
20	10	90
25	5	95

3.6.2 Characterisation of *O*-Coumaric Acid Using Fourier-Transform Infrared Spectroscopy

A chemical structure by means of existences of functional groups in the samples were identified using FTIR (Shimadzu, Model Nicolet Nexus 470, USA) technique with an attenuated total reflectance mode in the range between 4000 to 8000 cm^{-1} averaging 64scans at a resolution of $8cm^{-1}$ was operated for sample analysis.

The die and its components were ensured to be clean and the rubber seal was properly fixed in the groove of the die. About 2-3 mg of dried samples in the form of fine powder was transferred into the bore of cylinder and adjusted to be evenly distributed across the police face. The spectrum obtained on the screen was compared with standard and the spectra was printed.

3.7 One Factor at a Time Experiment of Ultrasonic Assisted Extraction

3.7.1 Effect of Extraction Temperature on Extraction Yield

The *G. sepium* leaves were prepared before the experiments by drying at temperature of30 to 70^{o}C until the moisture content was below 10%. The extraction was carried out byusing a sonicator (QSonica, Model Q500, US). The sonicator system comprises of three major components; generator to transfer AC line power to high frequency electrical energy, convertor to transfer electrical energy to mechanical vibration and probe to amplify and transmit the vibration. The extract temperature was regularly monitored using thermometer.

The solvent used in the extraction was methanol and the ratio of methanolto solid was 30:1 (ml: g) and the selected process duration was 30 minutes. The experimental conditions were closely examined from screening experiment using temperature ranges of 30, 35, 40, 45, 50, 55, 60, 65, 70 ^{o}C. The experiments were conducted in triplicates.

After the extraction, about 2 ml of sample was filtered using syringe filter for solid particlesremoval. Dilution of the filtrate was carried out by adding 950μl of methanol to 50μl of filtrate. The diluted extract (10.0 μl) was injected into HPLC system for analysis to determine its chemical profiles and concentrations of *o*-coumaric acid. The HPLC analysis was carried out following the procedures given in Section 3.4.3.

3.7.2 Effect of Ratio of Solvent to Solid (Rss) on Extraction Yield

The dried *G. sepium* leaves were prepared before the experiments by oven drying at fixtemperature of 50^{o}C for 30 minutes until the moisture content was below 10%. The extraction was carried out by using a sonicator system. The experimental conditions wereclosely examined for screening experiment using R_{ss} range values at 5:1, 10;1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 45:1 and 50:1 of solvent to solid ratio (ml/g). The experiments were conducted in triplicates.

After the extraction, about 2 ml of sample was filtered using syringe filter for solid particlesremoval. Dilution of the filtrate was carried out by adding 950μl of methanol to 50μl of filtrate. The diluted extract (10.0 μl) was injected into HPLC

system for analysis to determine its chemical profiles and concentrations of *o*-coumaric acid. The HPLC analysis was carried out following the procedures given in Section 3.6.1

3.7.3 Experiment for Effect of Extraction Time on Extraction Yield

The dried *G. sepium* leaves were prepared before the experiments by oven drying at temperature of 50ºC until the moisture content was below 10%. The extraction was carried out by using a sonicator system. The experimental conditions were closely examined for screening experiment using extraction time varies from 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 minutes. The experiments were conducted in triplicates.

After the extraction, about 2 ml of sample was filtered using syringe filter for solid particles removal. Dilution of the filtrate was carried out by adding 950µl of methanol to 50µl of filtrate. The diluted extract (10.0 µl) was injected into HPLC system for analysis to determine its chemical profiles and concentrations of *o*-coumaric acid. The HPLC analysis was carried out following the procedures given in Section 3.3.2.

3.7.4 Experiments for Effects of Temperature, Ratio of Solvent to Solid and Extraction Time on the Extraction Yield using Response Surface Methodology

The extraction solvent was methanol (80%) which was obtained from the previous works. The extraction was performed by ultrasonic equipment (QSonica, Model Q500, New York) at constant power of 130 W and frequency of 20 kHz.

The experiments were designed using and three parameters were selected for the optimized extraction parameters including extraction temperature, time and solvent to solid ratio. The chosen parameters and its level were specified according to the preliminary study as reported in Chapter 2.

The extraction was performed using a fixed solvent of 80 % methanol at 50°C for 30 mins. The extraction parameters were optimized using Response Surface Methodology and central-composite design (CCD). CCD was performed for three factors and five levels (-α, -1, 0, +1, +α), and the experimental datawas then fit with the second order models to optimize the responses. The experimental runs were randomized to measure the responses and suit with the appropriate regression mathematical equations. Table 3.4 summarizes the extraction parameters and the design codes at five levels. The independent variables; temperature, time and R_{ss}. levelwas labelled as A, B and C.

Table 3.4 : The employed variables in CCD experiment

Levels	**Independent Parameters**		
	Temperature (°C)	Time (min)	Solvent to Solid (mL/g)
-α	46.6	36.6	1.6
-1	50	40	5
0	55	45	10
+1	60	50	15
+α	63.4	53.4	18.4

Design expert software, version 13 (Stat-Ease Inc., Minneapolis, MN, USA) was used forthe RSM. This design required 17 runs and three replications were performed at each run number.

The actual data which is obtained from the experimental work (output data) were subjected to the regression analysis between the independent parameters. In the analysis, yield was set as the response variable (Y) and was a function of coded independent variables including temperature (A), time (B) and ratio of solvent to solid (C).

The response variable and independent variables was proposed using second-order polynomial model which was given as Equation 3.11:

$$Y = b_0 + b_1 A + b_2 B + b_{12} AB + b_{11} A^2 + b_{22} B^2 \quad (3.11)$$

where Y is the predicted response variable, b_O is the regression coefficient of intercept term, b_1 and b_2 are linear regression coefficients, b_{11} and b_{22} are squared regression coefficients and b_{12} is interaction regression coefficient. The data were analyzed by least square method to fit the model.

The analysis of variance (ANOVA) was used to evaluate the significance of each variable on the resulted model. The significance was determined statistically by computing the F-value and p-value ($p < 0.05$). The lack of fit measures how well the models fit the experimental data with the estimated values, and the presented value would be insignificant with a p-value > 0.5. A higher coefficient of determination denoted as R^2 (above 0.8) indicates that the model is precise and workable for best fitting the experimental values within the ranges of limit (Yang *et al.,* 2017).

Furthermore, the adjusted R^2 (Adj R^2) and predicted R^2 (Pred R^2) were calculated for model's accuracy. The difference between Pred R^2 should be reasonable (<0.2) and the Adj R_2 is important to demonstrate the high precision and reliability of the models' accuracy (Asfaram *et al.,* 2016; Yang *et al.,* 2017).

The three-dimensional (3D) plots of contour and surface were generated to visualize the relationship between the given values of each factor and predict the optimum factor conditions. The developed 3D plotswere analyzed for each response by keeping one response variable at its optimal value and then plotting the other two independent factors.

3.8 Fermentation Process for Bioconversion of *O*-Coumaric Acid to Dicoumarol

3.8.1 Strain Propagation and Culture Maintenance

The lyophilized ampoule was opened according to the enclosed instruction. From a singletest tube of 5mL sterile distilled water at, about 1.0mL of it was withdrawn with a sterile pipette and directly applied to the pellet. The solution was stirred to form a suspension. The suspension was aseptically retransferred into the test tube of sterile distilled water.

The test tube was let undisturbed at room temperature for overnight as rehydration mightincrease most of the fungal viability. The grown strains were aseptically transferred into potato dextrose broth (Oxoid, UK) and incubated for 18-24h at 30˚C. The sub culturing process was regularly performed in

500mL Erlenmeyer flask and incubated for 3-4 days at 30˚C.

3.8.2 Inoculum Preparation

An inoculum containing *A. fumigatus* NRRL 163 was prepared. Stock cultures were streaked on potato dextrose agar (Oxoid, UK) and incubated at 37^{o}C overnight. A single colony was removed from potato dextrose agar and inoculated into 100 mL of PDB in Erlenmeyer flasks (500 mL). Inoculated flasks were shaken vigorously at 200 rev/min using rotary shaker (Jiangsu Technology, Model HZ-300, China) for 10 to 12 hours at 37°C. The cell viability was tested and cell count was done using haemocytometer. The inoculum with minimum ~1.0 x 10^{7} CFU/mL was used as a standard inoculum for fermentation in shake flasks.

3.8.3 Medium Preparation

For fermentation process, two types of mediums were prepared. The complex medium containing 4 gL^{-1} of potatoes extract, 20 L^{-1} dextrose and 20 L^{-1} agars were set to pH of 5.1at 25°C.The defined medium which consists of 5 g L^{-1} leaf extract, 5 g L^{-1} D-glucose, $NaNO_3$ (6g), KCl (0.52g), $MgSO_4.7H_2O$ (0.59g), KH_2PO_4 (1.52g) and trace elements of $FeSO_4.7H_2O$ (4mg), $ZnSO_4.7H_2O$ (0.4mg) was added into 1L deionized water. The medium was set to pH 4.8 after sterilization as suggested by Bye and King (1970).

3.8.4 Leaves Surface Sterilization

The method used for surface sterilization was modified from the method used by Burgdorf*et al.* (2014). Leaf samples were rinsed with tap water followed by sterile distilled water. The leaves were placed in a 2 mL microtube with 1.5 mL 1.0% NaCl solution and 0.3 g of 0.1 mL acid washed beads (Sigma-Aldrich, USA). The tubes were spinned on a vortex(Scientific Industries, Model Bead Genie, USA) for 10 min. Samples were rinsed three times in 1 mL of sterile ultra-pure water until no odor presence. The leaves were stored in plastic bags and frozen at -80 °C before further processing.

3.8.5 Solid State Fermentation

Solid state fermentation (SSF) was used using grounded leaves powder as suggested by Nattha *et al.* (2013) with some modifications. A 250 mL Erlenmeyer flask was filled with 5g of grounded leaves powder and sterile distilled water with liquid to solid ratio to 5:1 (w/w), equating to moisture contents of 85%. Around 5mL of sucrose solution were addedinto each flask to enhance the fungal growth.

Under aseptic condition, about 10% (v/w) inoculum of *A. fumigatus* NRRL 163 was evenlypoured on the substrates. A sterilized spatula was used to mix the mixture of fresh leaves and grounded leaves powder for even inoculum distribution and the flask was cotton- pludged. The mixture was incubated in a static incubator (Memmert, Model IN 110, India) at 28 °C for up to 7 days with regular monitoring. The samples using only whole fresh leaves with the same liquid to solid ratio were used as control.

3.8.6 Optimization of Submerged Fermentation in Shake Flask

Submerged fermentation was conducted in 500 mL flasks with 200mL working volume. About 10% of inoculum size containing minimum of 1.0×10^6 spores/mL were inoculatedinto each flask and flasks were agitated at 250 rpm at 30 °C. Sampling was done at defined time intervals for viability test and biomass determination using dry cell weight.

The optimization method was carried out using single factor and can be divided into threestages. In the first stage, the selection of medium composition had been conducted in batch fermentation using shake flask culture. The two types of different medium compositions had been selected which were complex medium (Potato Dextrose) and defined medium containing 5 g L^{-1} D-glucose, $NaNO_3$ (6g), KCl (0.52g), $MgSO_4.7H_2O$ (0.59g), KH_2PO_4 (1.52g) and trace elements of $FeSO_4.7H_2O$ (4mg), $ZnSO_4.7H_2O$ (0.4mg). The optimum medium that produced high dicoumarol was selected to be used.

Then, the effect of initial concentration of glucose (0, 5, 10, 15, 20, 25 g g L^{-1}) and initial nitrogen concentration (0, 1, 2, 3, 4, 5 g L^{-1}) has been investigated using shake flask culture as suggested by Bazilah *et al.* (2010). The optimum parameters were selected from those with the highest yield of dicoumarol production. In the last stage, the fermentation data were used for the evaluation of growth kinetics of *A. fumigatus* NRRL 163. Several kinetics parameters such as maximum specific growth rate (μ_{max}), biomassyield ($Y_{x/s}$), product yield ($Y_{p/s}$) and growth associated constant (α) were determined.

The moulding hay in each flask obtained from SSF was shaken with 9ml of sterile waterand 1.0 ml of supernatant fluid was inoculated aseptically into 100mL fermentation medium under high aseptic condition. The values of temperature and agitation speed of orbital shaker were set according to the experimental design as 37˚C and 170 rpm, respectively. The other set of experiments were run using the same shake flask without inoculum as control.

3.8.7 Analytical Procedures

3.8.7.1 Cells Viability Test

Cell's viability was determined by comparing the colony forming unit (CFU) with total spores. For viability determination, harvested media was filtered through a compacted glass wool. The filtrate was centrifuged (Hettich, Model Micro 22R, Japan) at 10,000 rpmfor 10 minutes and the supernatant was discarded.

The resulting spore pellet in each centrifuge was resuspended in 5mL of distilled water and centrifuged again at 12,000 rpm for 10 minutes, discarding the supernatant. The pellet was removed from each centrifuge tube, spread in a petri dish and dried in desiccators (Model Phyrex, UK) with silica gel for 3 days.

The CFU numbers were determined by plating serial dilutions of conidial preparation onto potato dextrose agar. Prior to plating, dry conidial preparations were soaked in sterile distilled water for 2h and then grounded in a blender (Waring, Model TBB160E, UK) at maximum speed for 3 minutes.

The viable colonies counted directly from PDA and weredetermined by using Equation 3.12 (Houchmandzadeh and Ballet, 2023).

CFU (CFU/mL) = number of colony x 0.25mL sample x dilution number (3.12)

3.8.7.2 Determination of Spores Count

The number of spores was counted by using a haemocytometer (Model Neubauer, UK).The haemocytometer was initially cleaned using 70% ethanol. About 20 µl of the samplewas loaded into the grid made between the coverslip and the counting chamber of haemocytometer up to the groove's edges. The spores count was calculated under 10x magnification of microscope using Equation 3.13 (Wolk, *et al.,* 2000).

Spores count (spores/mL) = Average number of cells x dilution factor x 10^4 (3.13)

3.8.7.3 Determination of Cell Dry Weight

During the fermentation process, 10mL of culture sample was withdrawn from the sampling line at each of 12h interval time for analysis. The sample was centrifuged (Hettich, Model Micro 22R, Japan) at 10, 000 x g for 10 minutes. The supernatant was used for the analysis of pH profile while the cell pellet was used for the dry cell weight determination.

The cell concentration in term of dry cell weight was determined by filtration and oven drying method. About 2mL of broth sample was filtered through a pre-weighted 0.2µm Whatman No. 1 filter membrane by using a vacuum filtration set. The cells were then dried in an oven at 80˚C until a constant

weight has achieved. The dry cell weight (X) was calculated as follows (Molino and Joao, 2020);

$$X_{(g/L)} = \frac{W_f - W_i}{V} \quad (3.4)$$

Where

X = *Dry cell weight (g/L)*

W_f = *Wight of dry filter paper + cells (g)*

W_i = *Weight of dry filter paper (g)*

V = *Sample volume*

3.9 Bioconversion of *O*-Coumaric Acid into Dicoumarol

3.9.1 Separation and Purification of Dicoumarol

The fermentation broth was incubated on orbital shaker for 72 hours with regular sampling at 12 hours' interval for culture broth analysis. The samples were filtered free using pore size 11μm filter paper (Whatman™ 1001-125 Grade 1, US) from mycelial debris and shaken mechanically for 20min with an equal volume of chloroform as suggested by Bellis *et al.* (1967). The chloroform layer was separated and evaporated to about l ml for media separation. The separated liquid was used for sample purification using HPLC.

Dicoumarol fraction was collected through HPLC and compared to the standard. For fraction peak validation, the tube was located at the end of waste pipe. The fraction was collected from the beginning of its chromatographic peak and sample collection was stopped at the end of the peak. The collected

sample of diroumarol was re-injected for three times into HPLC to get the best match of peak to its standard. All the obtained chromatograms were then compared to its standard and its retention times were recorded.

3.9.2 Identification and Characterisation of Dicoumarol

The incubated samples media were extracted with chloroform, then chromatographed and quantitatively analysed for dicoumarol by using HPLC, FTIR and LCMS/MS.

3.9.2.1 Identification of Dicoumarol Using High Performance Liquid Chromatography

High performance liquid chromatography (Shimadzu, Model SPD 20A, US) was used to analyse the product qualitatively and quantitatively. HPLC is precise and accurate in analysis and quality control of plant materials and extracts. The system consists of UV- VIS detector with wavelength range from 190 to 700nm.

Dicoumarol was selected as quality indicator in this work. It is because the compound was reported to be potential anticoagulant derived from *G. sepium* leaf extract as being discussed in section 2.1.3. The standard of dicoumarol acid was purchased from Sigma-Aldrich (USA).

A ZORBAX Eclipse Plus C18 column (250mm x 4.6mm, 5 µm particle size, USA) was used as stationary phase. The mobile phase was in gradient mode and consists of methanol and sodium acetate (75:25 v/v) at pH 6.0. The

changes of mobile phase content during the analysis were shown in Table 3.5. The combination of mobile phase was selected for better separation. The detection wavelength was 330nm as the compound is sensitive to the wavelength. The experiments were conducted in triplicate for the better reliability.

Table 3.5 : Mobile phase changes in HPLC analysis for dicoumarol

Time (min)	Sodium Acetate, $C_2H_3NaO_2$ (25%)	Methanol, MeOH (75%)
5	80	20
10	40	60
15	20	80
20	10	90
25	5	95

3.9.2.2 Characterisation of Dicoumarol Using Fourier Transform Infrared Spectroscopy

A chemical structure by means of existences of functional groups in the samples were identified using Fourier Transform Infrared Spectroscopy, FTIR (Thermo Nicolet, Model Nexus 470, USA) technique with an attenuated total reflectance mode in the range between 400 to 5000 cm^{-1} averaging 64 scans at a resolution of 8cm^{-1} was operated for sample analysis.

FTIR spectra were established using KBr discs in the range of 375-7000 cm^{-1} spectral range. Leaf dried sample in the form of thin film was placed across a hole in the sample holder.The die and its components were ensured to be clean and the rubber seal was properly fixed in the groove of the die. About 2-3 mg of dried samples in the form of fine powder was transferred into the bore of cylinder and adjusted to be evenly distributed across thepolice face. The

spectrum obtained on the screen was compared with the standard and the spectra was printed.

3.9.2.3 Characterisation of Dicoumarol Using Liquid High Performance of Chromatography Mass Spectrometry-Mass Spectometry

Dicoumarol was further identified using high performance liquid chromatography connected to a triple quadrupole detector of HPLC-MS/MS system (IET, Model AB Sciex4000 QTrap, USA). A Zorbax Eclipse XDB-C18 column (Narrow-Bore, 150 x 2.1 mm ID, particle size 5µ m, Agilent Technologies, USA) was used.

The conditions were initially attained in 3 minutes and the system was stabilized for 4 minutes. The system was operated at room temperature with flow rate at 0.3mL/ min. Dicoumarol compound was measured in positive and negative electrospray ionization (ESI+/ESI) using a mass range between 50-600 m/z due to it high signal intensity of precursor ions. The optimum collision energies were set between 5-50 eV to get at leasttwo intense fragment ions. The acquisition was performed in SRM mode using two transitions from precursor ions to product ions for each compound identification.

The mobile phase composition consisted of binary mixtures with 5mM of Ammonium Formate in MeOH and 5mM of ammonium formate in water. Gradient elution during anlaysis were shown in Table 3.6.

Table 3.6 : Mobile phase changes in HPLC-MS/MS analysis for dicoumarol

Time (min)	Ammonium Formate in Methanol, NH4HC02 -MeOH (20%)	Ammonium Formate in Water, NH4HC02-H20 (80%)
3	20	80
8	50	50
14	70	30
18	90	10

3.10 In Vitro Rodenticides Anticoagulant Activity

3.10.1 Chemicals and Reagents

Prothrombin Time (PT) and Activated Partial Thromboplastin (APTT) reagents and all K_3EDTA tubes and 3.2% sodium citrate tubes were purchased from Thermo Fisher Scientific, US. Phosphate Buffer Saline was purchased from Sigma Aldrich, USA.

3.10.2 Specimen Collection and Processing

Blood specimens were collected through venipuncture method and withdrawn in 3.2% sodium citrate tubes with a collection ratio of 3:1, 5:1, 7:1 and 9:1 blood to anticoagulant. The sodium citrate tubes were filled completely and the specimens were gently mixed after collection. All the specimens were brought to the lab within 4-8 hours and kept at 15-25˚C to prevent blood haemolysis. The specimens were then capped centrifuged at 3000 rpm for 10 minutes using Centrifuge (Sigma Aldrich, Model Hettich EBA 20, US) toachieve platlet-poor plasma.

3.10.3 Prothrombin Time (PT) Test

The action in extrinsic pathway of dicoumarol was evaluated by PT test. The test was carried out using commercial PT reagent kits. About 90 µL of plasma was mixed with 10µL of and incubated at 37°C for 5 min. Then, 200 µL of PT assay reagent (sodium chloride) pre-warmed at 37°C for 10 min was added and the clotting time was recorded by a digital coagulometer (Pioway, Model CL-2000, China). Plasma alone was used as control in the absence of anticoagulant activity. Dicoumarol standard (1µg/µL) was used as positive control.

3.10.4 Activated Partial Thromboplastin Time (APTT) Test

The action in intrinsic and common pathways of dicoumarol were evaluated by aPTT test. The test was carried out using commercial aPTT reagent kits. About 90 µL plasma was mixed with 10 µL of samples and incubated at 37°C for 5 min. Then, about pre-warmed 25 mM aPTT reagent (calcium chloride) was added and incubated at 37°C for 2 min. The clotting time was recorded by a digital coagulometer (Pioway, Model CL-2000, China). Plasma alone was used as control in the absence of anticoagulant activity. Dicoumarol standard (1µg/µL) was used as positive control.

CHAPTER 4

DRYING AND OPTIMISATION OF ULTRASONIC ASSISTED EXTRACTION FOR RECOVERY OF O-COUMARIC ACID

4.1 Introduction

Drying process is governed by principle of heat and mass transfer simultaneously (Chupawa *et al.,* 2015). As the materials undergone drying process, excess moisture is removed; hence the water activity of the wet material is reduced. In this research, oven drying was used with the goals to remove excess moisture from the plant material by concentrating the target compounds and preventing microbial growth during storage.

After drying process, solid liquid extraction was used and it started with the solvent penetration into the solid matrix, solute dissolution in the solvents, solute diffusion out of the solid matrix and collection of the extracted solutes (Zhang et *al.,* 2018). The solid material of *G. sepium* leaves that contains the *o*-coumaric acid was brought into contact with the solvent. Then, the soluble compound in the solid material was dissolved in the solvent, forming a solution before it undergone the separation of the solution from the insoluble residue through centrifugation technique. Lastly, the desired compound of *o*-coumaric acid was recovered and purified from the solution using evaporation technique.

4.2 Drying of Gliricidia sepium Leaves

4.2.1 Effect of Drying Temperature on Extraction Yield

The effect of drying temperatures (40, 50, 60, 70 and 80°C) on extraction yield was studied to determine the ideal temperature for *G. sepium* leaves drying for the optimum *o*-coumaric acid crude extraction. The temperature ranges selected suits to the most of the literaturesinvolving the thin layer behaviour of plant-based materials (Osman *et al.,* 2019; Turan *etal.,* 2019; Muajffar *et al.,* 2018 and Janjai *et al.,* 2011).

The optimum temperature was then selected based on the highest yield of *o*-coumaric acid obtained from the HPLC results as shownin Figure 4.1. The figure shows the comparison of chemical profiles between standard and dried leaves under different drying temperatures. The HPLC chromatograms (Figure A.1) revealed that the drying temperatures were found to influence the extraction yield.

There was a major peak detected in both fresh and driedleaves by HPLC at wavelength 290nm and it was identified as *o*-coumaric acid by comparing their retention times at about 2.78 min. The peak of *o*-coumaric acid in chromatogram of fresh leaves was lower in comparison to those in dried leaves. The difference is caused by the drying process which has caused the physical and chemical changes in the *G. sepium* leaves.

The highest yield of *o*-coumaric acid was obtained at 50°C with followed by 40 and the yield started to show the decreasing pattern at 60 to 80°C of drying

temperatures. The peak noticed at the retention time of 3.5 min was identified as solvent peak.

The results also showed that the concentration of *o*-coumaric acid was lower in fresh leaves but reverse trend was observed in all the dried products. The results revealed that the dryingprocess at different temperatures had caused the changes of *o*-coumaric acid concentration. The rupture of cell wall of the leaves during the drying process exposes the internal leaves matrix and allows the solvent to penetrate effectively into the solid matrix and extract thedesired phytochemicals.

Similar trends were reported in the studies using tomato slices by Osman *et al*. (2019) in the range of drying temperatures at 40-80°C.Figure 4.1 shows the recovery of *o*-coumaric acid in fresh leaves and the dried *G. sepium* leaves of different temperatures including 40, 50, 60, 70 and 80°C. The recovery of phytochemical was calculated as the percentage of ratio of phytochemical recovered to the mass of driedleaves. The concentration of *o*-coumaric acid was calculated from their peak areas by using the calibration curves developed from the standard.

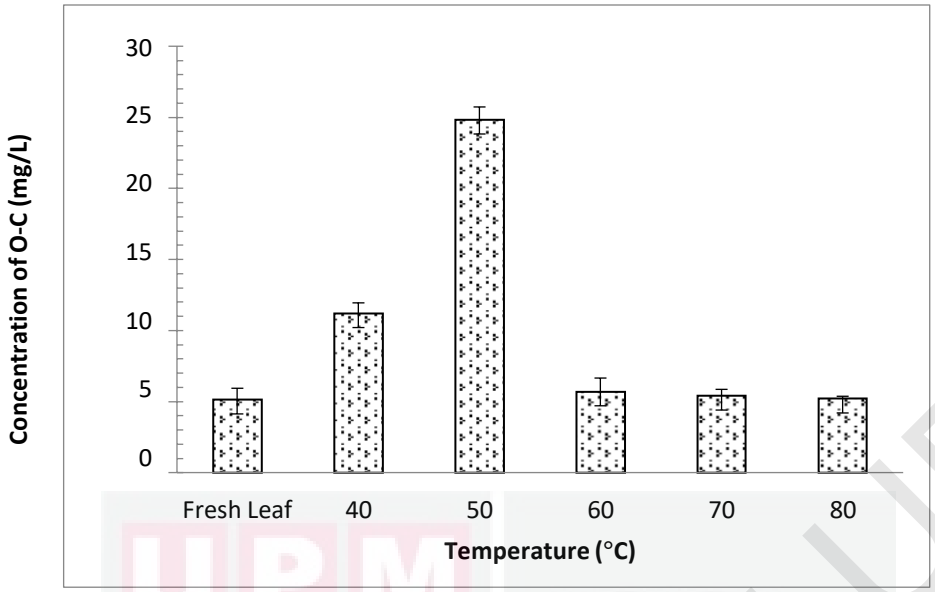


Figure 4.1 : Recovery of *o*-coumaric acid in the fresh leaves and dried leaves from different drying temperatures

The recovery of *o*-coumaric acid of fresh leaves was used as control. The recovery of *o*-coumaric acid increased with the extraction temperature at 50°C then started decreasing. However, the leaves were not properly dried at 40°C as it only could extract half than 50°C. The phenomenonwas might due to the low drying heat which limits the removal of moisture content. The increasing yield in 50°C (25 mg/L) was resulted from the increase of solubility of *o*-coumaric acid in methanol at higher extraction temperature.

The percentage of increase dropped after 50°C because the system was approaching the equilibrium state in which saturation of *o*-coumaric acid concentration happened and the compound started to degenerate. This implies thatthe critical extraction temperature for *o*-coumaric acid is 50˚C.

Significant reduction of *o*-coumaric acid recovery was observed as the drying temperature rose to 60ºC as compared to 50ºC with 71.86% reduction in *o*-

coumaric acid recovery. This result demonstrates that *o*-coumaric acid initiated the thermal decomposition above 60 ^{o}C temperature. The extracted yield showed the decreasing pattern after drying temperature of 60 ^{o}C and it remains slightly decreasing for higher temperatures.

By lowering moisture content of 12-10% durimg drying process, there was a marked increase in *o*-coumaric acid concentration as shown in Figure 4.1 comparing fresh leaves with 40 ^{o}C dried leaves (54.3% increase of *o*-coumaric acid recovery).

The boiling point of *o*-coumaric acid is 348.0±17.0 °C at 760 mmHg. From ANOVA analysis, the concentrations of *o*-coumaric acid dried at 50^{o}C were significantly highest ($p < 0.05$) among the studied temperatures. The optimum drying temperature was 50^{o}C as the highest concentrations of *o*-coumaric acid was obtained. Thus, the temperature at 50^{o}C was suitable for drying of *G. sepium* leaves to avoid the *o*-coumaric acid degradation during the process.

4.2.2 Effect of Drying Temperature on Drying Kinetics

G. sepium leaves were dried until there was no change in sample weight, signifying the equilibrium of moisture content. The drying times according to experimental conditions from the initial 75% to a final 0% were 413, 173, 127, 66 and 60min respectively for the temperature ranges of 40 to 80°C. Simultaneously, Figure 4.3 illustrates the changes in moisture ratio of *G. sepium* leaves with drying time and drying rate respective to the moisture content.

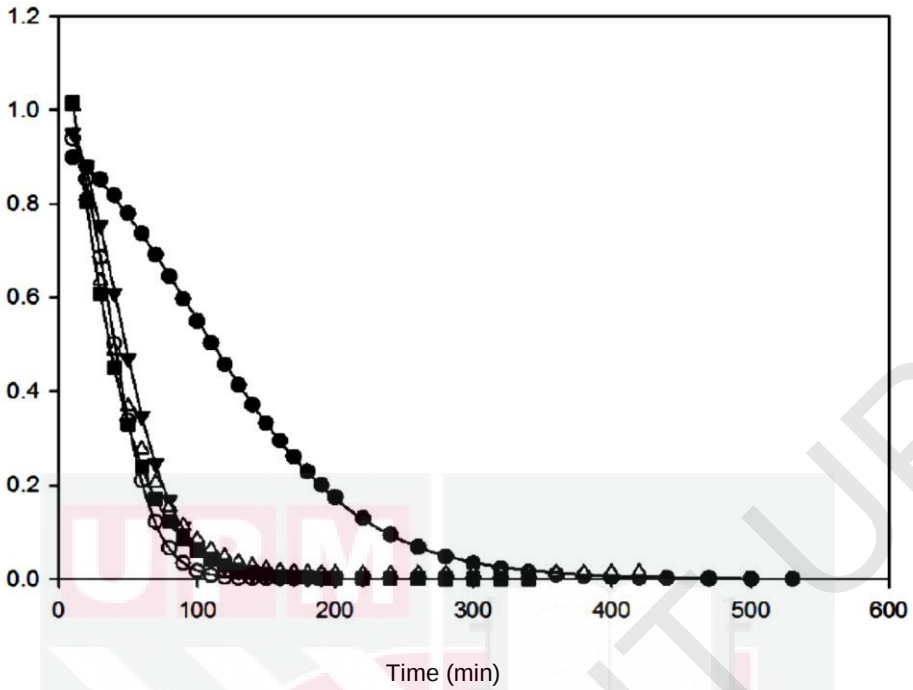


Figure 4.2 : Dimensionless moisture ratio vs time (min) for temperatures 40°C, 50°C, 60°C, 70°C and 80°C

From Figure 4.3, moisture ratio reduced continuously as the drying time increased. It alsoshows that the leaves could not properly remove the moisture content through drying at 40°C as proven in the result of *o*-coumaric acid recovery in Figure 4.2. There is no constant rate drying period detected in in these curves due to quick removal of moisture and all the drying operation occurred in the falling rate period. Thi *et al.,* (2019) reported a similar pattern in the drying of mint leaves. This is because the leafy materials which are thin take a very short period of time to be heated up.

Continuous decrease in moisture ratio signifies that diffusion has governed the internal mass transfer. Pisalkar *et al.* (2014) stated that higher drying temperature decreased themoisture ratio quicker as the heat supply rate to the leaves increased and due to the acceleration of moisture migration. It can be

seen that there was a great variation in drying time for the drying air temperatures at 40°C as compared to above temperatures.

Moisture reduction found to be temperature dependent and slow at lower temperature and took longer as compared to drying at higher temperatures. Experimental results showed that drying temperature is effective parameter for the drying of *G. sepium* leaves. The drying rates versus residual moisture content have been represented in Figure 4.3.

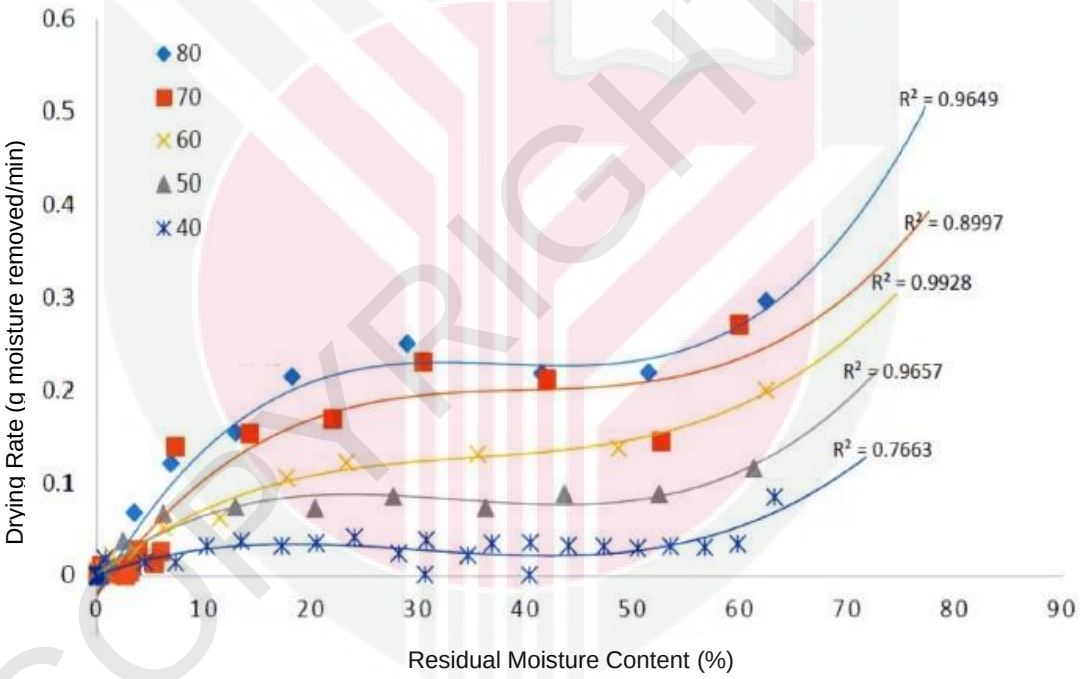


Figure 4.3 : The drying rate for different drying temperatures

The curves demonstrated smooth diffusion-controlled drying behaviour under all run temperatures as been supported by Kumar *et al.* (2013). It is apparent that drying rate decreases continuously with moisture content. Moreover, an important influence of air- drying temperature on drying rate could be observed

in these curves. An increase of drying rate was observed with the increase of air-drying temperature. The highest values of drying rate were obtained at 0.297 g moisture min $^{-1}$ at drying temperature of 80°C. The drying in falling rate period shows that that internal mass transfer has occurred by internal diffusion. The results are also in agreement with the observations of earlier research on leafy products by Doymaz *et al.* (2010); Brooks *et al.* (2008) and Singh *et al.* (2007).

4.2.3 Evaluation on Thin Layer Models

Moisture content significantly influences the quality and shelf life of dried *G. sepium* leaves. The equilibrium moisture content was achieved after no change in the weight sample was observed. Throughout the drying process, the time required to reach the equilibrium moisture content for the *G. sepium* leaves was 10.0, 3.5, 2.5, 2.0 and 2.25 hours at drying temperatures of 40, 50, 60, 70 and 80°C respectively. The findings are parallel to the drying theory where shorter drying time is required when higher drying temperature is applied on the plant materials.

The moisture ratio data of *G. sepium* leaves dried at different temperatures were fitted into the thin layer drying models. By using SOLVER in Microsoft Excel 2013, the parameters of all the thin layer models were solved for R^2 and RMSE. The coefficient of correlation and results of statistical analyses are listed in Table 4.1. In all cases, the R^2 values for the mathematical models were greater than 0.95, indicating a good fit.

The RMSE values obtained were also very low, as low as 0.05. By evaluating the resultsthat have highest values of R^2 and lowest values of RMSE, Midilli *et al.* model was the best model to fit the drying kinetics of 40°C, 50°C, 70°C and 80°C. Experiment data for only 60°C on the other hand was best fitted by data predicted from Page model. However,60°C also showed substantial fit towards Midilli-Kucuk model. Thus, Midilli *et al.* model may be assumed to represent the thin layer drying behaviour of *G. sepium* leaves in an oven dryer. Similar findings were reported for drying of thin layer rough rice (Onwude *et al*., 2016) bay leaves (Doymaz, 2014). The plot of experimental data and the best-fitmodel are shown in Figure 4.4.

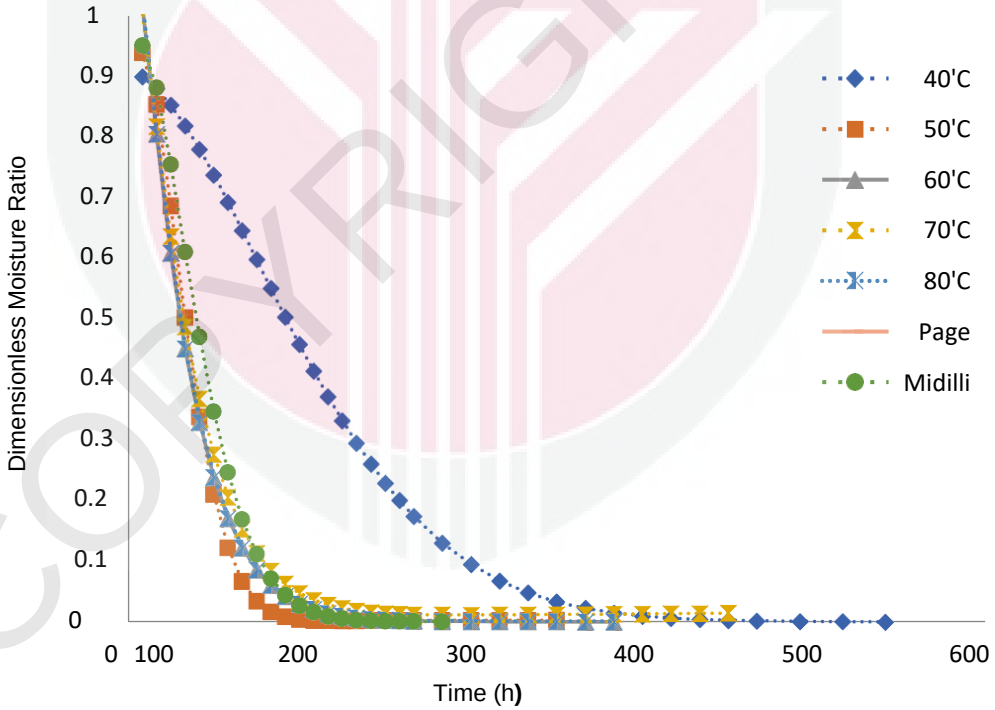


Figure 4.4 : Comparison between the experimental data with the selected model-Midilli-Kucuk model for different temperatures

However, it can be observed that there was no significant improvement in R^2 and RMSEvalues using more complicated models like Logarithmic and Midilli

et al. which contain three and four drying constants respectively in comparison to the much simpler Lewis model. In this study, the difference of R^2 values between the most suitable model and theleast suitable model was less than 0.36%. Since Midilli-Kucuk model showed the closestR^2 value to unity for all studied temperatures, it was chosen to be most applicable in thedrying of G. *sepium* leaves. It was also observed that the solution obtained for the drying constants for Modified Page model was identical to Lewis model in all the drying temperatures except 40°C. This was because the drying constant of Modified Page model – n was approximately 1 in all the solutions. This indicated that Lewis model was sufficientto describe the process instead of adding an extra drying constant – n.

As shown in Table 4.1, t is clear that changes of temperature have affected the constantsand coefficient values of all models. For example, as Page model coefficients (k and n) for each drying air temperature were calculated, an increase in air temperature resulted in an increase in the constants and coefficients of the model. For example, when air temperature rose from 40, 50, 60, 70 and 80°C, k values showed increasing pattern withk = 0.0009, 0.0034, 0.0069, 0.256 and 0.0286 respectively. The pattern remains same tothe others models.

By using SOLVER in Microsoft Excel 2019, the parameters of all the thin layer models as listed in Table 3.2 were solved for correlation coefficient, R^2 values close to unity withthe lowest values of Root Mean Square Error (RMSE). The values of evaluated criteria for all the models are given in the Table 4.1. It

showed that R^2 values for all experiments. From ANOVA analysis, the concentrations of *o*-coumaric acid from *G. sepium* leaves dried at 50˚C were significantly highest ($p < 0.05$) among the studied temperatures. The most suitable drying temperature was 50˚C as the highest concentrations of *o*-coumaric acid obtained. Thus, thetemperatures ranging from 40 to 50˚C were suitable for drying of *G. sepium* leaves to avoid the degradation of *o*-coumaric acid.

Table 4.1 : The values of coefficient correlation, RMSE and drying constants for all models

Drying air temperature (°C)		40	50	60	70	80
Model name						
Page	k	0.0009	0.0034	0.0069	0.0256	0.0286
	n	1.4296	1.4733	1.3776	1.2162	1.3394
	R^2	0.9857	0.9927	0.9994	0.9914	0.9957
	RMSE	0.0024	0.0016	0.0001	0.0015	0.3834
Modified Page	k	0.1052	0.1835	0.2062	0.2740	0.2814
	n	0.0710	0.1239	0.1393	0.1851	0.1901
	R^2	0.9542	0.9656	0.9831	0.9860	0.9834
	RMSE	0.0077	0.0076	0.0034	0.0025	0.3787
Lewis	k	0.0078	0.0227	0.0287	0.0507	0.0535
	R^2	0.9542	0.9656	0.9831	0.9860	0.9834
	RMSE	0.0077	0.0075	0.0034	0.0025	0.3787
Logaritmic	k	0.0085	0.0242	0.0305	0.0531	0.0562
	a	1.1326	1.0789	1.0711	1.0320	1.0567
	b	0	0	0	0.0046	0
	R^2	0.9676	0.9709	0.9871	0.9870	0.9860
	RMSE	0.0055	0.0064	0.0026	0.0023	0.3797
Midilli	a	0.9077	0.9514	0.9392	1.0084	1.0179
	b	0	0	1.216E-05	9.725E-05	0
	k	0.0002	0.0019	0.0343	0.0381	
	n	1.7204	1.6071	0.0019	1.1303	1.1311
	R^2	0.9895	0.9941	1.7058	0.9918	0.9990
	RMSE	0.0018	0.0013	0.9956	0.0015	0.0002
				0.0009		

*a, b, k, n are values of drying constants for all applied models

The effects of five different drying temperatures on the drying kinetics and quality of *G. sepium* leaves were investigated successfully in this work. The recovery of *o*-coumaric acid was determined from HPLC results. The optimum drying temperature for *G. sepium* leaves was found to be 50°C judging from the findings within the studied temperatures.

Decrease of *o*-coumaric acid recovery was observed when the leaves dried at 60°C. The drying kinetics data indicates that the increase of drying temperature shortens the drying time. The drying happened mostly in the falling rate period. Midilli model was the most suitablemodel to describe the drying process of *G. sepium* leaves under the studied temperatures. For further result validation, the correlation between Midilli predicted and experimental moisture ratio was plotted with high similarity (R^2 = 0.9955) as presented in Figure 4.5

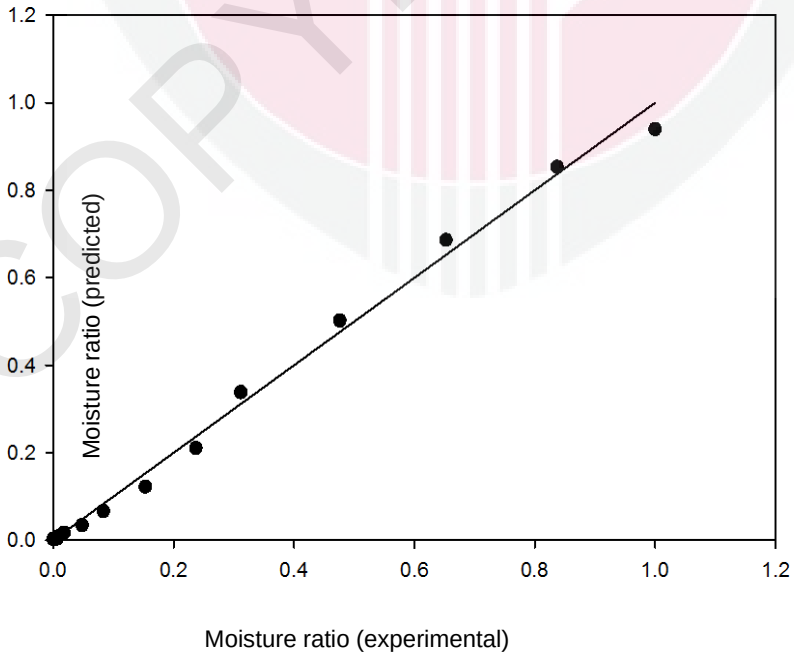


Figure 4.5 : Correlation between Midilli predicted and experimental moisture ratio

4.3 Physical and Chemical Analysis of Dried *G. sepium* Leaves

4.3.1 Scanning Electron Micrograph of *G. sepium* Leaves

The dried *G. sepium* leaves were prepared as described in Section 3.5.1. The structural analysis of plant material is particularly important in the optimization of extraction process. Figure 4.6 shows the scanning electron micrograph of the samples at room temperature. The comparable physical modification of *G. sepium* leaves were noticed according to theextraction process used.

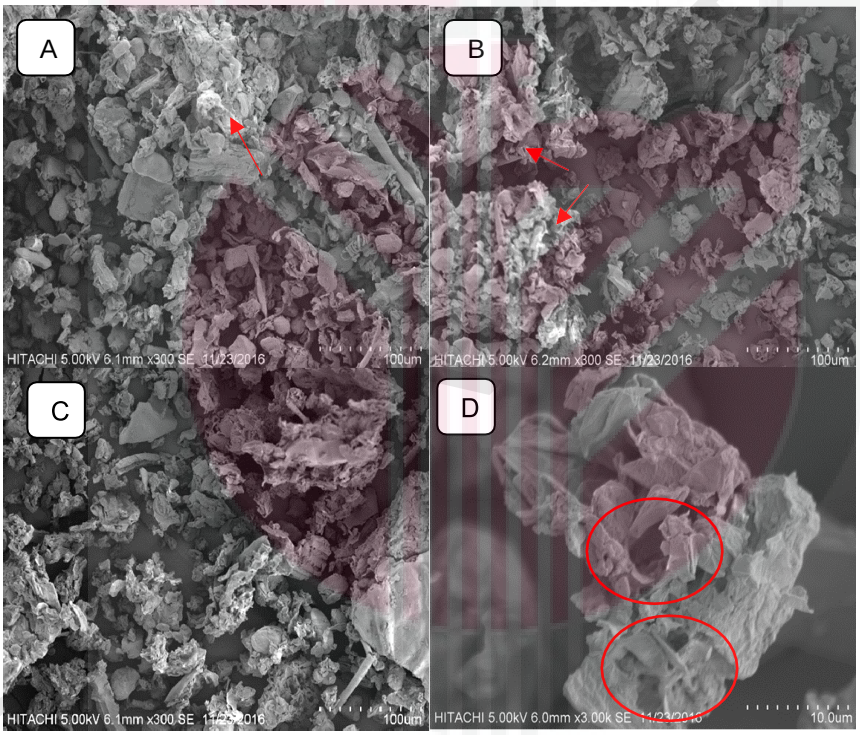


Figure 4.6 : Effect of ultrasound power on *G. sepium* leaves. SEM microscopic observation of *G. sepium* leaves at room temperature; (A) untreated raw material, (B) SE, (C, D) UAE (US probe, 20kHz, 15m

The untreated leaves appear filled and intact. The cell structures of conventional extracted leaves were intact but emptied due to transfer of cell content within the solvent. A gradual degradation of cell walls was obtained after

UAE which were affected at various degrees and duration of times. During UAE, there was quick fragmentation of *G. sepium* leaves in the first few minutes of sonication. It was comparable when the leaves did not seem impacted during conventional extraction performed by SE. The cells structures of UAE were destructed to undefined shapes. This finding could be assumed that such cell destruction fragmentation favoured accessibility to the solvent reaction.

4.3.2 Particle Size Distribution of *G. sepium* Leaves

G. sepium grounded leaves were initially prepared through sieve filteration to measure the particle size distribution. The distribution of particle size is an initial key in understanding both physical and chemical properties. In Figure 4.7, it shows that the particle size distributions were in the range of 200-300 µm for both leaves from UAE and SE methods.

The particle size distribution was calculated by volume-based. The result indicated that 9.6% of the size distribution was in the category of 181.97 to 239.88 µm, which means the total volume of all particles with diameters in this range represents 9.6% of the total volume of all leaves explants. Comparatively, it was 1.8% lower in size distribution in the category of 208.93 to 239.88 µm for SE. The range of particle size distribution of d≤ 250 µm are preferable in most leafy materials prior to extraction process as being reported by some previous works on rosemary leaves (Rodriguez *et al.*, 2012); spirulina leaves, (Dey *et al.,* 2013) and stevia (Jaitak *et al.,* 2009).

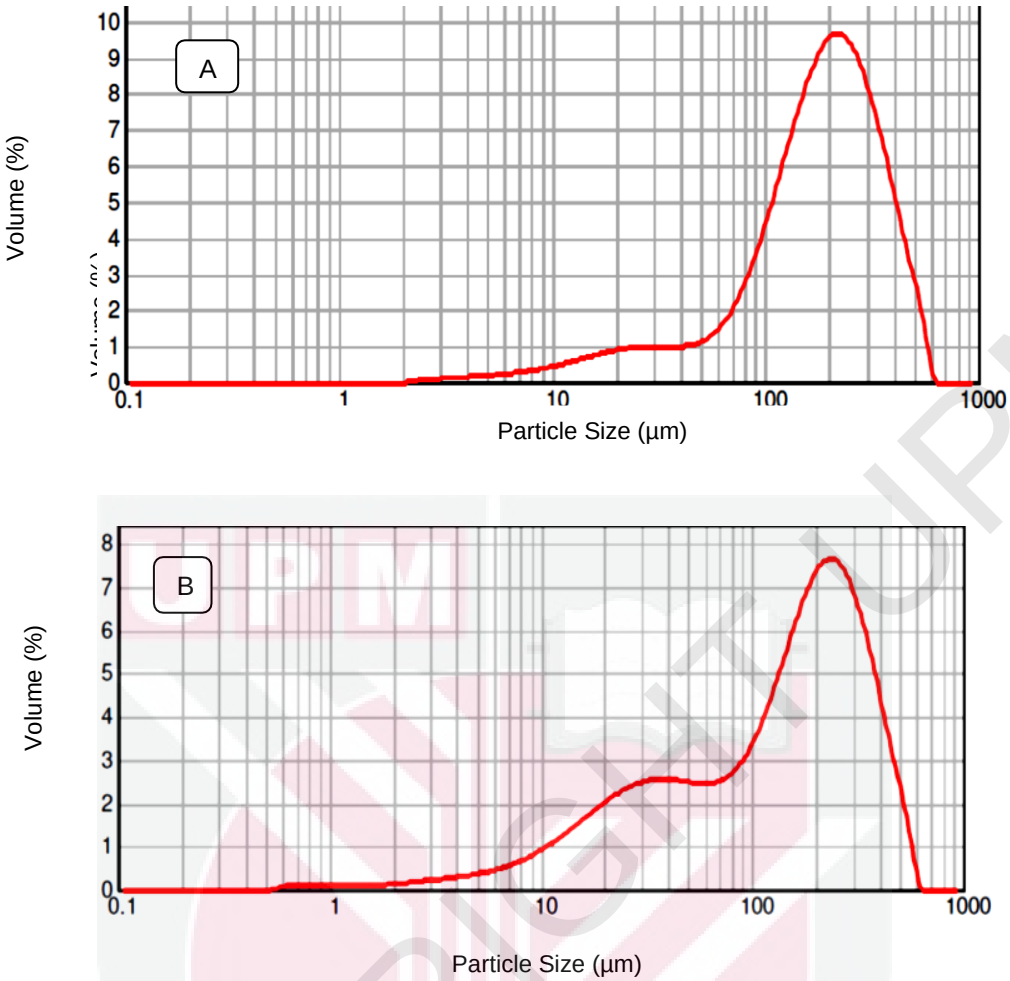


Figure 4.7 : The average particle size of leaf by (A) UAE, (B)

Ultrasonic cavitation has led to a fragmentation of friable solids. The cells fragmentation could be due to inter-collision between particles and from shockwaves created from collapsing cavitation bubbles in the solvent (Farid *et al.,* 2017). The reduction in particle size distribution by ultrasound action can lead to direct consequence of increase surfacearea of the solid materials which resulted in higher mass transfer and increase extractionrate and yield.

The specific surface area of *G. sepium* residues from UAE and SE methods were 0.1852m^2 g^{-1} and 0.0782 m^2 g^{-1} respectively. UAE showed higher surface area than SE indicating the finer particles size for better extraction result.

According to Zhang *et al.* (2018), the extraction efficiency will be enhanced if smaller particles size is exposed due to the enhanced penetration of solvents and diffusion of solutes. By creating finer particles in UAE as compared to SE, it increases the surface area which reduces the physical barriersto dissolution, thus making the quicker extraction process. This concluded that UAE technique reduces the inner particles size hence, increases the extraction efficiency andrecovery yield.

4.3.3 Thermogravimetric Analysis of *G. sepium* Leaves

The thermo gram for *G. sepium* showed five distinct segments as shown in Figure 4.8. Segments 1-3 indicate weight loss in nitrogen while segments 4-5 indicate weight loss in air. The lighter volatile materials noticed initially at temperature of 71.25°C. The release of medium volatile materials initiated at temperature of 241.53°C and last up to 325.16°C.

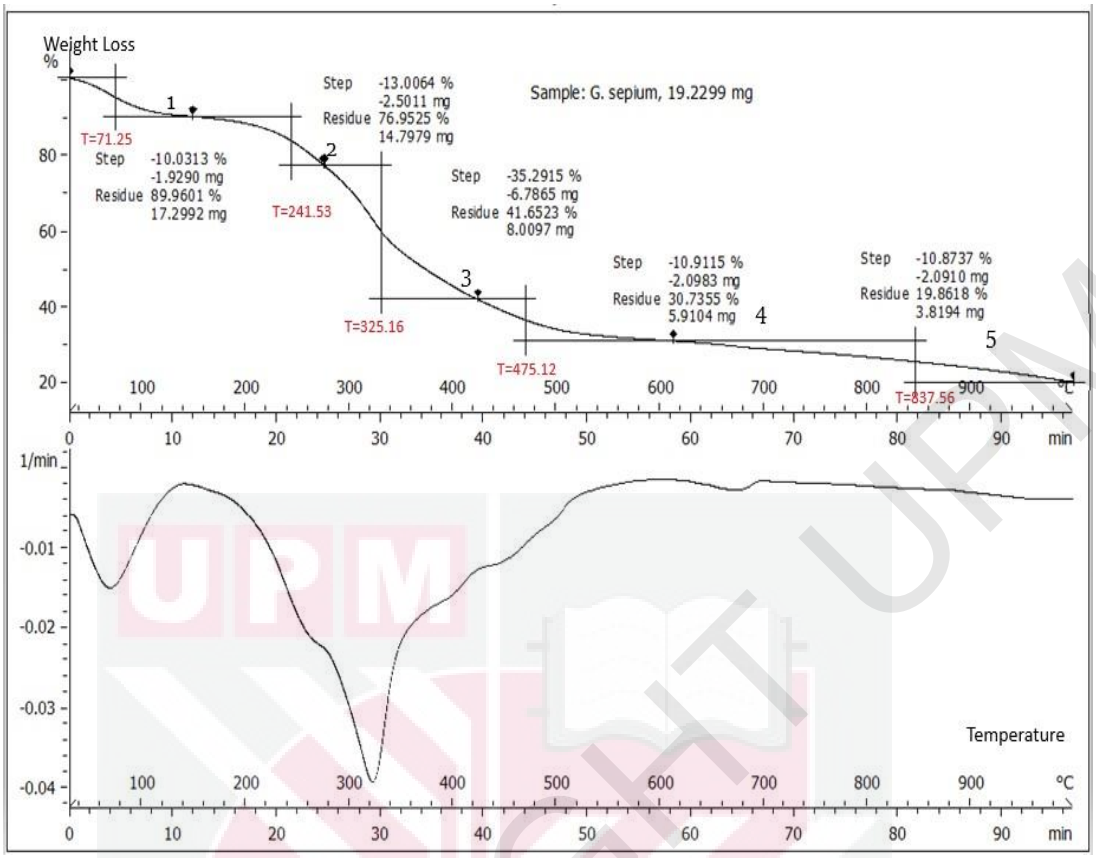


Figure 4.8 : Thermogravimetric curve for *G. sepium* leaf residues

The release of heavier volatile materials starts at temperature of 325.16°C and continued up to 475.12°C. The fixed carbon decomposition occurred at 837.56°C until the final heating temperature. In TGA curve implies that the observed weight loss is due to the presence of volatile materials and it showed that the final residue was 3.8194 mg (19.8618%) in a form of ash.

4.3.4 Proximate Analysis of *G. sepium* Leaves

Proximate analysis of dried *G. sepium* leaves is shown in Table 4.2. The initial moisturecontent of fresh leaves was recorded as 81.37% (wet basis).

Table 4.2 : Proximate analysis of dried *G. sepium* leaves

Sample	Mass (mg)	Moisture (%)	Volatile Matter (%)	Fixed Carbon (%)	Ash (%)
G. sepium	19.2299	10.0313	59.2094	10.8737	19.862

Moisture content is of important interest since it corresponds to one of the main criteria for the selection of plant extraction. Thermal conversion technology requires plant extractwith low moisture content for the optimum extraction yield. The higher moisture content indicates the poor drying technique and hence affects the yield of bioactive compound tobe extracted.

The extraction materials exhibit a wide range of moisture content for extraction purposes ranging 5-8% for oil recovery (Olumekun *et al.,* 2021) and up to 10-12% for leafy materials for bioactive extraction (Truong *et al.,* 2019). From table 4.2, it showed that *G. sepium* dried leaves contain 10.03% of moisture content and hence most suitable to serve as extraction materials. This also parallels to Ellis *et al.* (2006) which stated that the minumum moisture content for extraction of bioactive compound in several plants are optimum at 10% and lower.

4.3.5 Effective Moisture Diffusivity

As stated in section 4.2.2, all the drying of *G. sepium* leaves using oven dryer occurred in falling rate period only and the drying process is controlled by liquid diffusion. The effective (D_{eff}) was calculated using slope derived from the linear regression of ln MR against drying time (min) as shown in Figure 4.9.

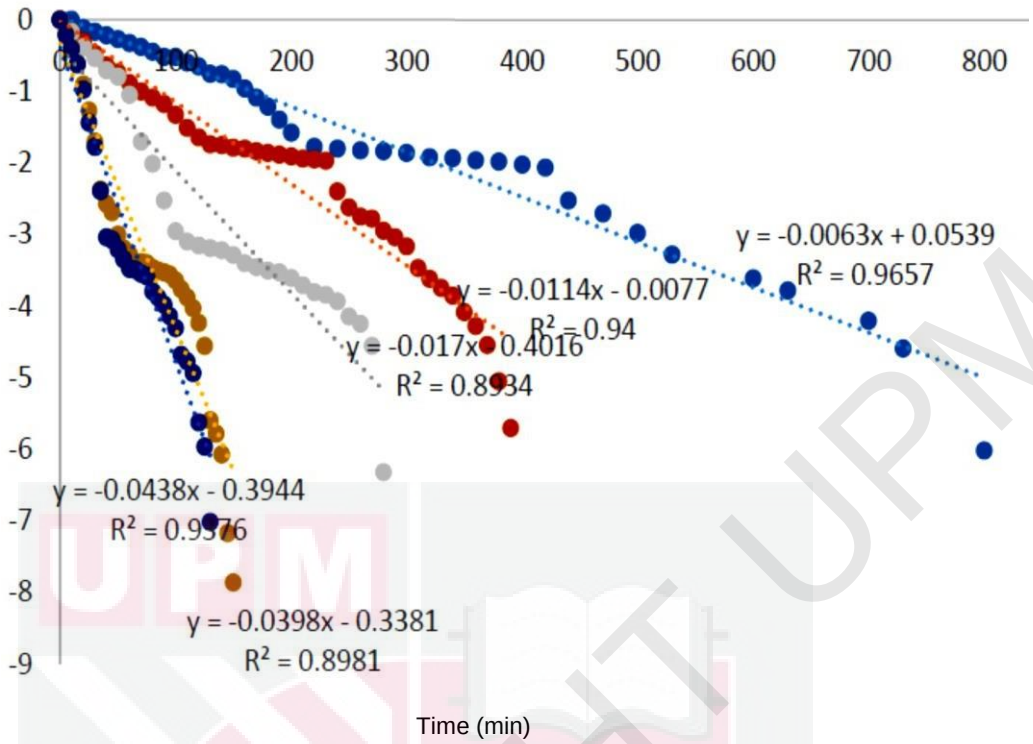


Figure 4.9 : Linear regression of ln (MR) against drying time for all drying temperatures

D_{eff} of drying process of *G. sepium* leaves for each drying temperature was summarizedin Table 4.3. It showed the linear relationship between D_{eff} and drying temperature. D_{eff} increases with the increasing drying temperature. D_{eff} was increased from 2.06650×10^{-11} to 1.43671×10^{-10} as shown below;

Table 4.3 : Effective diffusivity for oven drying of G. *sepium* leaves

Temp (˚C)	Deff x 10^{-11} (m^2 s^{-1})
40	2.06650
50	3.73939
60	5.57628
70	13.0551
80	14.3671

This result also similar to the previous study on prickly pear by Amira *et al.* (2014) as shereported that D_{eff} of moisture transfer using the Fick's Law at three drying temperatures (40, 50, and 60°C) were in the range of 2.53×10^{-10} to $7.6 \times 10^{-10} m^2 s^{-1}$, respectively. The variations of ln (D_{eff}) versus (1/T) for *G. Sepium* leaf was shown in Figure 4.10.

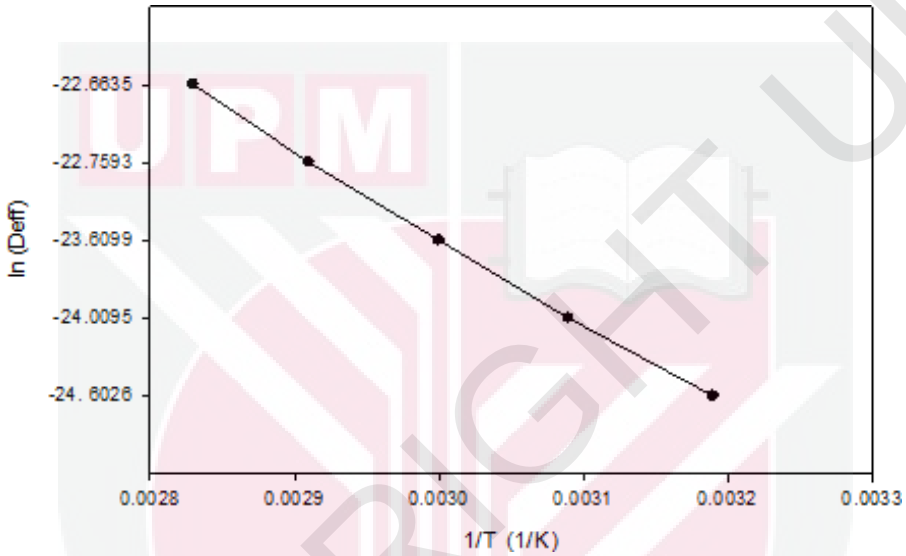


Figure 4.10 : Logarithms variation of moisture diffusivity with (1/T)

Analysis of the experimental data revealed the existence of linear relationships between these parameters. Correlation coefficient (R^2) of the fitted lines with experimental data was obtained as 0.9661. However, this method is applicable with an assumed constant D_{eff} throughout the whole drying process. Indeed, D_{eff} is rarely constant but varies with moisture content, temperature and spatial coordinates (Amira *et.al*, 2014).

4.4 Ultrasonic Assisted Extraction of *O*-Coumaric Acid from *G. sepium* Leaves

4.4.1 Effect of Solvent Types and Concentration on Extraction Yield

There were five types of solvents used in the experiments including hexane (Hex), ethylacetate (EA), chloroform (Cloro), methanol (MetOH) and pure water (H_2O). The extraction was carried out by using a heating mantle with temperature of 50°C, ratio of solvent to solid (R_{ss}) at 30:1 (ml: g) for 30 minutes. The effect of solvent types on the extraction method was investigated as the choice of an appropriate solvent is the most important parameter for the optimized extraction of specific constituents. The solvent polarity in increasing order was Hex < EA < Chloro < MetOH < H_2O. The result showed that, methanol MetOH revelead the highest yield concentration in both extraction methods as compared to the rest solvents as shown in Figures 4.11 and 4.12.

Methanol has a polarity index of 5.1. Methanol is mostly used for extraction various polarcompounds (Truong *et al.,* 2019; Altemimi *et al.*, 2017) but certain group of non-polar and unreadily soluble compounds are fairly soluble in methanol. Moreover, methanol amongall other solvents has low boiling point of just 65°C. Thus, extraction and recovery of *o*-coumaric acid was easy accomplished by both extraction systems. *o*-coumaric acid was semi-standard non polarcompound and it was slightly soluble in water but well dissolved in methanol as polar solvent.

Another point to the finding was the molecule of methanol which consists in a single atomof a tetrahedral carbon, linked to 3 hydrogens and a -OH group.

The -OH group is the polar group and the three hydrogens, the water-insoluble hydrocarbon chain. This contributes to the ability of methanol to dissolve polar molecules and also non-polar ones. Madiha *et al*. (2017) also reported the similar finding on the study for cinnamon leaves as methanol showed the highest extraction yield (27.49 ± 1.59%) among the rest solventsof chloroform, hexane and ethanol. Figures A.1 and A.2 show the comparison of HPLC chromatograms of UAE and SE respectively.

From all selected solvents, methanol with the second highest polarity showed the highestyield with 66.32% difference as 28.95 and 9.75 mg L^{-1} respectively for both UAE and SEdepicted in Figure 4.11. UAE is obviously promotes better advantages against SE in termof acoustic cavitation effects. The propagation of ultrasonic waves provides a greater solvent penetration into the sample matrix, increasing the contact between the sample and the solvent and improving the mass transfer rates (Duarte, 2014).

UAE also promotes better cell ruptures within the *G. sepium* leaves for better solvent penetration. In term of solvent consumption, the conventional extraction consumed about100 to 200 ml whereas only 50 ml of solvent used in UAE method. It showed 4 times decrease in solvent consumption. From both extraction methods, UAE was found to be the best as compared to SE.

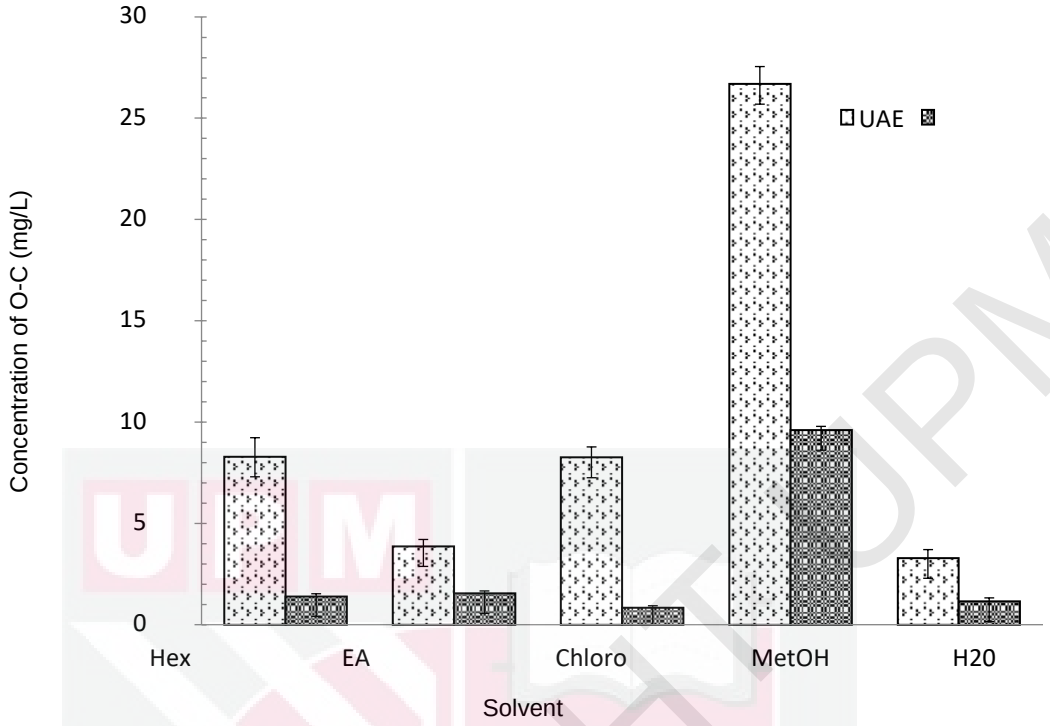


Figure 4.11 : Comparison of yield concentration from different solvent types andextraction systems

From Figure 4.12, it showed the increasing and decreasing graph pattern for methanol concentration. It indicated that methanol alone could suppress the concentration of *o*-coumaric acid to be extracted. The 80% of methanol was the ideal as solvent in extraction for *o*-coumaric acid compound from *G. sepium* leaves using UAE under optimal parameters (temperature of 50 °C, ratio of solvent to solid (R_{ss}) at 30:1 (ml: g) and time for 30 minutes. The reason for this range could be due to the *o*-coumaric acid bioactive compound belongs to large chemical class,so solvent system for extraction is required, which can dissolve the large quantity of medium polar compound. This also parallel to the parameters used in the HPLC system.

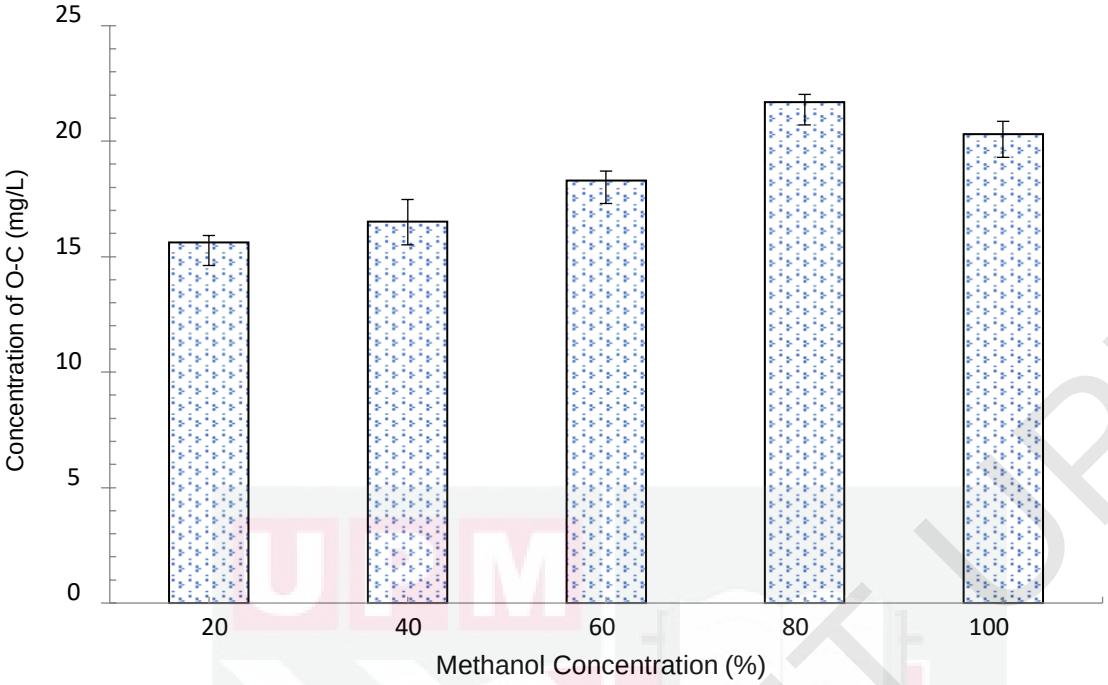


Figure 4.12 : Yield concentration from different methanol concentration

The presence of higher concentration of methanol in the extraction solution creates a larger concentration gradient. This acts as a driving force for higher diffusion of solvent into the plant cells, thereby, improving the overall mass transfer of the system (Ameena, 2014). Moreover, increased amount of methanol enhances the contact area between thesolvent and the solute, thus, improving the solubility of the phenolic compounds from within the plant cells (Moorthy *et al.,* 2017; Xu *et al.,* 2017). Prasad *et al*. (2011) also claimed the solubility and extractability of polar phenolic compounds are better with polar solvents.

4.4.2 Effect of Temperature, Time and Rss on the Extraction Yield

Single experiment indicated that the recovery of *o*-coumaric acid increased with the extraction temperature. However, the recovery of *o*-coumaric acid

decreased after 60°C. The increase was resulted from the increase of solubility of *o*-coumaric acid in water at higher extraction temperature. The percentage of increase dropped after 60ºC because the system was approaching the equilibrium state in which saturation of *o*-coumaric acid occurred.

This observation indicates that *o*-coumaric acid concentration degraded at those high temperatures more than 60ºC as the concentration gradually decreased when the temperature was raised to 70 and 80ºC. It was also reported by Tan *et al.* (2013) that most phenolic compounds are heat sensible; hence higher temperature must be respected to avoid degradation of thermo sensitive phenolic compounds.

Most importantly, 80% methanol (v/v) was used for the extraction, thus the temperature must not exceed its boiling points of 64.7ºC. A very high temperature may lead to methanol evaporation from the aqueous methanol solution and it will subsequently change methanol-water ratio. The recovery of *o*-coumaric acid increased when the extraction time was raised from 35 to 45 minutes but decreased as higher time of 50 and 60 minutes were applied. The recovery of *o*-coumaric acid also decreased with the increasing of solvent-to-solid ratio. It showed that the lower ratio gives the higher yield of *o*-coumaric acid.

4.5 Optimization of Ultrasonic Assisted Extraction using Response Surface Methodology

4.5.1 Response Surface Methodology, Central Composite Randomized Design

The actual and predicted results of the response variables (dependent variables) were presented in Table 4.4. The extracted *o*-coumaric acid content varied from 9.53 to 36.14 mg L^{-1} based on different extraction conditions. Based on the ranges, the highest crude extract yield was obtained at run 5 with 47°C, 45 min and 10:1 g/mL solvent to solid ratio. The variablesare located near the centre point, assuming a value near to the optimum point for both responses (Ferreira *et al.,* 2007).

4.5.2 Model Fitting Summary

Multiple regression analysis and Pareto analysis of variance (ANOVA) were used to observe the adequacy and fitness of the developed models as presented in Table 4.4. Considering the results for single factor test, response surfaces analysis and the experimental values, the date of linear regression and binomial fitting were analysed. The regression model parameters ofthe sum of squares, degree of freedom (df), mean square, Fisher value (F-value) and p-value were also presented to evaluate the significance of the study for the respective surface quadratic models. The F-value with a small p-value (<0.0001) indicated that the result is significant and important to the response study (Mojerlou *et al.,* 2018).

Table 4.4 : Analysis of variance of the established quadratic equation mode

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > p	
Model	1076.55	9	119.62	2252.07	< 0.0001	significant
A-Temp	683.47	1	683.47	14582.09	< 0.0001	
B-Time	1.84	1	1.84	39.32	0.0004	
C-R_{ss}	120.8	1	120.48	2570.45	< 0.0001	
AB20.93	20.93	1	20.93	446.66	< 0.0001	
AC31.51	31.51	1	31.51	672.36	< 0.0001	
BC 8.05	8.05	1	8.05	171.76	< 0.0001	
A^2 85.89	85.89	1	85.89	1832.41	< 0.0001	
B^2 36.38	36.38	1	36.38	776.26	< 0.0001	
C^2	1.84	1	1.84	39.29	0.0004	
Residual	0.33	7	0.047			
Lack of Fit	0.15	2	0.077	2.23	0.2029	not significant
Pure Error	0.17	5	0.035			
Cor Total	1076.88	16				

The ANOVA results and regression coefficients were presented in Table 4.4 indicates that the contribution of the quadratic model was significant ($p < 0.05$). The Model F-valueof 2552.07 implies the model is significant. The values of Prob > F which less than 0.0500indicate model terms are significant. Thus, in this study A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms.

The fitness of the model was also investigated using the lack-of-fit test ($p > 0.05$), indicating the suitability of models to predict variations accurately (Prasad *et al.,* 2011). In this study, the Lack of Fit F-value of 2.23 implies the Lack of Fit is not significant relativeto the pure error. There is a 20.29% chance that the Lack of Fit F-value this large couldoccur due to noise. The non-significant lack of fit was good as the model was well fit.

The second-order polynomial model was solved using RSM was given as Equation 4.1;

$$Y= 15.37-7.84A +0.50B – 4.08C -1.91 AB + 2.34 AC – 1.18 BC + 2.60A^2 – 2.21B^2 + 0.5C^2 \quad (4.1)$$

The factors were coded as +α for the high level to predict the crude extract yield, while the low levels were coded as - α. These coded equations could identify the relative impact of the factors by comparing the coefficients of the estimated factors. The positive sign forthe coefficient value indicates that the tested variables have a synergistic effect on the responses. In contrast, a negative sign denotes an inverse impact on the responses. Themodels in Equation 4.1 was satisfied to predict the *o*-coumaric acid concentration within the specified parameters.

As shown in Equation 4.1, the *o*-coumaric acid concentration increased with decreasing of temperature and R_{ss}, as well as increasing value in extraction time. The *o*-coumaric acid concentration was also increased due to increasing in the interaction between temperature and R_{ss}, with decreasing in the interaction between temperature- time and an interaction between time- R_{ss}. The factors were proven to have a significant effect on the response yield.

The model gave a satisfactory fit with the experimental data because R^2 showed a valueof 0.9997. This means that the model could explain about 99% of the total variability withthe studied range. Figure 4.16 shows the comparison between the predicted versus actual data of yields. The plot indicates high level

of agreement between the experimentaland predicted data. Thus, this model can be used to calculate the yield of extraction with the studied range.

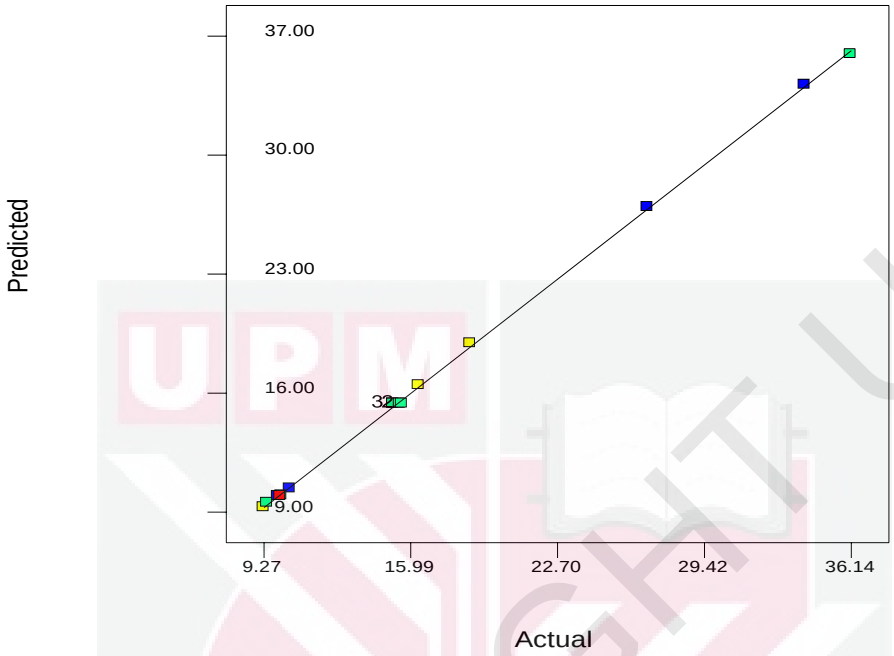


Figure 4.13 : Relationship between the experimental and predicted data

The relationship was determined based on the coefficient of determination (R^2) value. The lower the standard deviation values and the closer R^2 was to unity, the closer the predicted value was to the actual number (Samani, 2016). R^2 was regarded as high if it was greater than 80%, indicating an adequate conformity between the predicted and actual values.

As could be seen in Figure 4.13, the predicted value versus actual for the *o*-coumaric acid concentration showed that most of the values were well distributed along the regressionline, resulting in a better fit with high accuracy with R^2 value of 0.9997. As a result, the model exhibits a good correlation between the actual and predicted values, as the R^2 values correspond to be

greater than 0.9.

4.5.3 Effect of Extraction Parameters on Extraction Yield

The response plots of extraction yield were generated by varying two variables at a time. The evaluation of the linear terms from Equation 4.1 revealed that extraction time to have significant positive influence on dicoumarol. On the contrary, both extraction temperature and R_{ss} had significantly negative effects on the response. Interaction between extraction temperatures to R_{ss} displayed a significantly positive effect. The plots are in good agreement with regression analysis as the extraction time seems to have a slight positive effect on the recovery yield of *o*-coumaric acid.

The negative linear influence of R_{ss} was clearly noticed with maximum recovery of *o*-coumaric acid at 10:1 mL/g. The negative effect of extraction temperature was also clearly visible in Figure 4.14 as increasing temperature results in lower yield concentration. R_{ss} was found to have a more profound impact for the response. The surface plot indicated that the lower value of R_{ss} was preferable for *G. sepium* leaf extraction.

Figure 4.14 shows the response surface plots constructed based on the fitted model. The plots were constructed with one variable was set at a medium level and the other two were set within the experimental range. Figure 4.14 (a) shows the plot for variables of temperature and time with a medium level of time. As stated in Table 4.5, temperature was significant ($p < 0.0001$), while time was not significant ($p = 0.0004$).

An increase in temperature to 60°C would increase the yield of *o*-coumaric acid. This would increase the number of cavitation bubbles, leading to a greater solid–solvent contact area, thus improving of solvent diffusivity with an increase in the desorption of desired compound. This effect was, however, reduced when the temperature was near to the boiling point of the solventat 65°C.

Figure 4.14 (b) shows the plot for temperature and solvent-to-solid ratio with a medium level of temperature. Time was significant ($p < 0.0001$) in the study, and a decrease in of time would increase the *o*-coumaric acid concentration. This implies that the production of *o*-coumaric acid yielddepends on the time of extraction. UAE method offers shorter extraction time than SE. A longer exposure to the ultrasound may result in free radical production within the solvent,causing cell rupture. Lastly, Figure 4.14(c) shows the plot for R_{ss} and time with a mediumlevel of R_{ss}, where both R_{ss} and time were significant. An increase in R_{ss} and a decreasein time would increase the yield of *o*-coumaric acid, while a change in R_{ss} did not have much of an effect on the *o*-coumaric acid concentration.

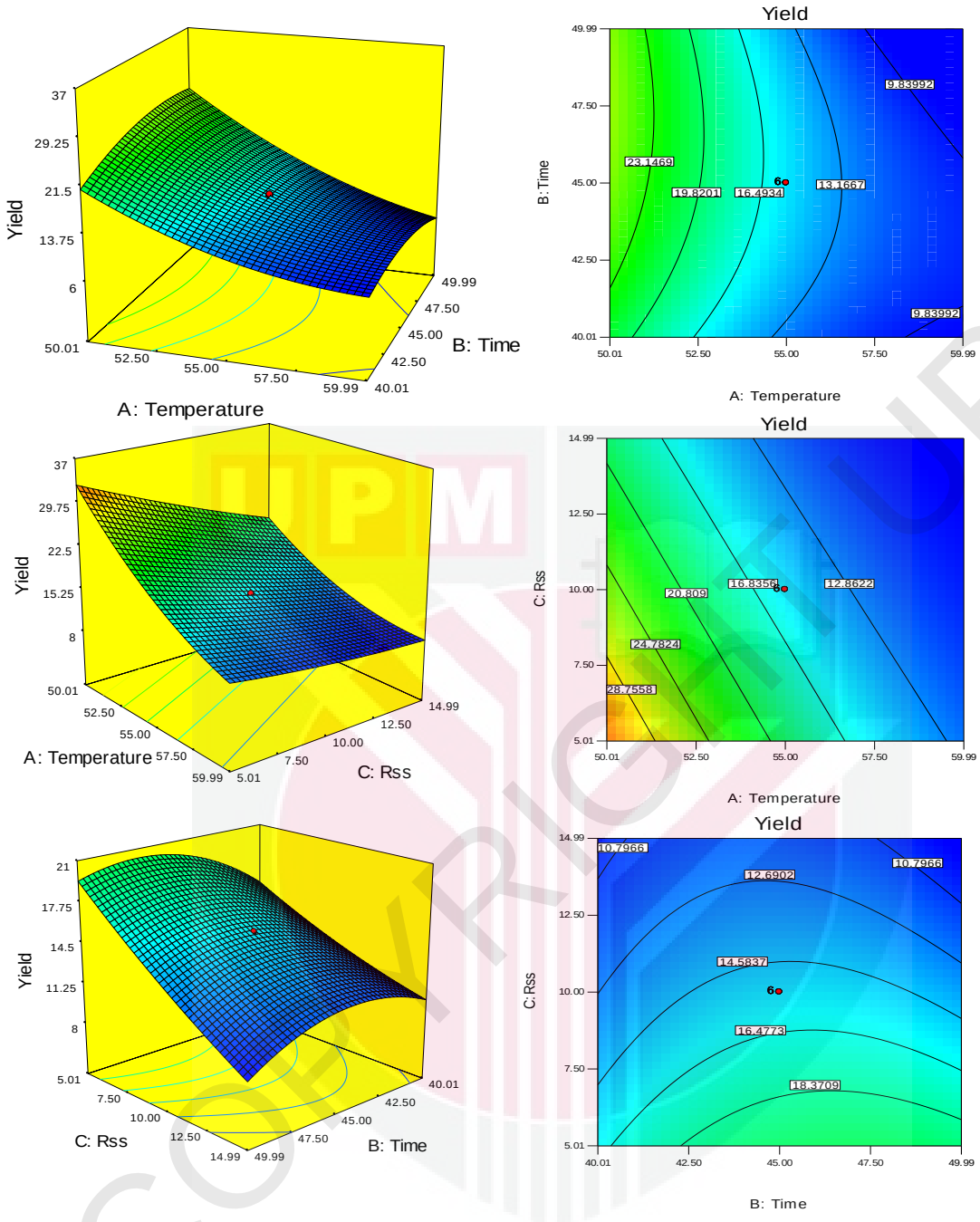


Figure 4.14 : Response surface and contour plots for the effect of independent variables on extraction yield of *o*-coumaric acid. A: Interaction between temperature (°C) and time (min), B: Temperature (°C) and Rss (mLg-1), C: Rss (mLg-1) and time (min)

4.5.4 Verification of Optimum Extraction Parameters

An optimum UAE conditions for obtaining maximum *o*-coumaric acid content in the extract were determined. All the factors and responses with the respective high-limit and low-limit experimental region were considered as listed in Table 4.5 to satisfy the criteria defined for the optimum operations. The predictive values matched well with experimental values obtained using optimum extraction parameters, which were confirmed with a good regression coefficient R^2 = 0.9997.

Table 4.5 : Significance test table of regression coefficient

Std. Dev	0.22	**R-Squared**	0.9997
Mean	16.68	**Adj R-Squared**	0.9993
C.V %	1.30	**Pred R-Squared**	0.9941
PRESS	6.39	**Adeq Precision**	160.449

Based on statistical analysis, the developed model was found to be significant at an F-value of 2552.07. High values of R^2 (0.9997) and adjusted R^2 (0.9941) indicates high degree of correlation. The predicted correlation coefficient (0.9993) was also determined to be of high significance. Adeq Precision measures the signal to noise ratio and a ratio greater than 4 is highly desirable. The ratio of 160.499 obtained in this study indicates it was an adequate signal. In addition, values of coefficient of variation (CV = 1.30%) and adequate precision further indicates the ability of the deduced model to define the relation between the extraction variables and the response of *o*-coumaric acid.

Table 4.6 : Comparison between the experimental and predicted values for theresponse variables

Parameters	Optimum Values		
Temperature (°C)		47	
Time (min)		45.00	
R_{ss} (mL/g)		10:1	
Responses	Experimental	Predicted	Error
Concentration of *o*-coumaric acid (mg/L)	36.14±0.015	35.92	0.006

Table 4.6 shows the experimental results of the actual run and the results from the modelfittings. The results obtained from the actual run were consistent and close to the valuespredicted by the model equation with only a small error. This condition showed a good accuracy for the prediction. The quadratic model predicted the optimal *o*-coumaric acid concentrationwas 36.14 mg/L with the generated error of only 0.006%. The desirability value was achieved at a high value, indicating the high precision of the regression model.

It was concluded that the best optimization conditions using the UAE method lies on extraction temperature of 47°C, extraction time of 45 min and R_{ss} of 10:1 mg L^{-1} resulted to *o*-coumaric acid concentration of 36.14 ±0.015 mgL^{-1}. These optimize conditions could be considered as optimum as well as feasible conditions.

4.5.5 Validation for Precision and Accuracy

The precision of the developed method was evaluated in terms of repeatability and intermediate precision. From the assessment of repeatability, three set of experiments on extractions were carried out under the optimum conditions on the same day while for intermediate precision about three sets of extractions

daily were carried out for three consecutive days. The coefficient of variation (CV) of the two levels of precision was summarized in Table 4.7.

Table 4.7 : Assessment of repeatability and intermediate precision

Bioactive Compound	Precision (CV, %)		Recovery (%)
	Repeatability	Intermediate Precision	
o-coumaric acid	0.61	1.23	86

The CV values were all satisfactorily for both repeatability and intermediate precision. Referring to AOAC, the acceptable limit for precision is ±10%. Hence, the developed UAE with optimum parameters was adequate to be considered as a precise extraction method.

4.6 Extraction Kinetics of *G. sepium* Leaf and Modelling

4.6.1 Variation of Extraction Yield with Time under Different Temperatures

The extraction rate of *o*-coumaric acid from *G. sepium* leaf was studied as a function of temperature. For each run, according to the optimum parameters, extraction process was carried out with 1g fine size leaf by using 80% methanol as solvent using UAE extraction method. The extraction rate at four different temperatures (45, 50, 60 and 65˚C) is presented in Figure 4.15 by plotting C_t (concentration of *o*-coumaric acid in the solution at any time in mg/L) versus time in min.

By increasing the extraction temperature, extraction duration can be reduced as the reaction occurs faster. The final concentration will also increase with

temperature due to classic thermodynamic effect of temperature on solubilisation phenolic compound inside the material. It means, as the temperature increases, the greater the diffusion coefficientand solubility of *o*-coumaric acid in the solvent thus, improving the rate of extraction.

As shown in the Figure 4.15, around 20% of *o*-coumaric acid was obtained in the first hour of extraction. The amount of *o*-coumaric acid extracted after one hour was 22.85, 25.9, 28.65, 29.55 and 31.9 mg/L for the extraction temperatures of 45, 50, 55, 60 and 65˚C respectively. For all studied temperatures, the extraction rate was highly fast at the beginning of the operation followed by the slowing rate for the remaining of extraction period.

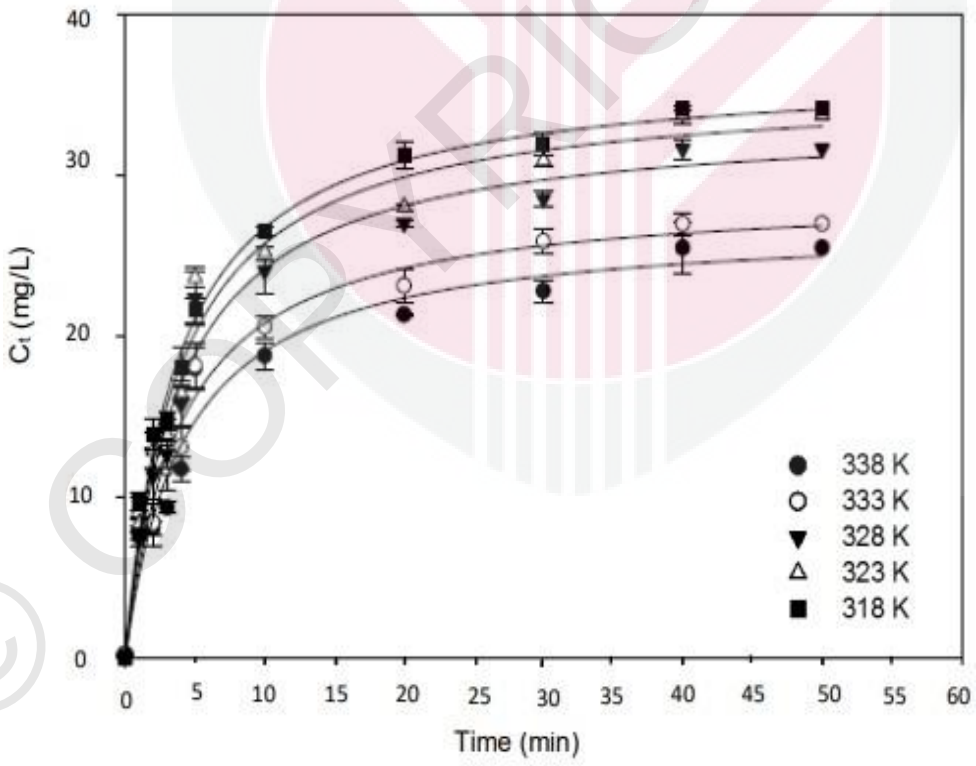


Figure 4.15 : Concentration of extracted *o*-coumaric acid versus time at different temperaturesin ultrasonic-assisted extraction extraction of *G. sepium* leaf

The behaviour of *o*-coumaric acid concentration could be visualized occur in two stages. Initially, the *o*-coumaric acid concentration was seen to rapidly increase followed by slow diffusive stage, beforereaching a constant value. The first stage involves the penetration, swelling of solvent into the outer plant surface and followed by the dissolution of soluble solutes into the solvent (Goula 2013; Mitic *et al.,* 2021).

The presented extraction behavior can also be related to a higher concentration gradientdeveloped during the initial stage and resulting in a rapid mass transfer rate and dissolution of soluble components into the liquid phase. This phenomenon was also similarly reported by Poojary and Passamonti (2015) in the study of lycopene that extracted from tomatoes. This current study examined the first stage process that occurred during the first 10 mins, before starting a slow diffusion stage when entering 30mins of the extraction process. The equilibrium stage was reached when there was no further improvement of the yield. The process probably involves external diffusion with approximate transfer of the residual yield at a lower velocity.

The concentration of *o*-coumaric acid remains unchanged when the extraction time proceeded from 50 minutes above and reached the equilibrium concentration. Kusuma and Mahfud (2017) reported the phenomena of solubilization and partition at the equilibrium phase, in which the solute is transferred at a constant velocity. Fick's second law of diffusion described that the solid and bulk solution would develop an equilibrium concentration after a particular duration (Lau *et al.,* 2015).

When any raw materials are dipped into a solvent, the bioactive compounds inside the material are solubilised and get extracted quickly inducing a fast increase in the extraction arte. As *o*-coumaric acid concentration is low in the solvent at the beginning of the extraction process, the compound diffuses rapidly from the leaf surface to the liquid phase. As the time passing by, the extraction rate becomes slower. This trend is due to the external diffusion phenomenon, which means diffusion takes place through stagnant liquid film around the solid particles. It is also due to the increase of *o*-coumaric acid concentration in the solvent, the diffusion rate decreases and the extraction rate slower consequently. When the maximum *o*-coumaric acid concentration is obtained, the yield level remains invariable even by extending the reaction time.

4.6.2 Extraction Kinetics Modeling

Three empirical equations such as first-rate order, second-rate order and Peleg model equations were applied to fit with the experimental data of *o*-coumaric acid extraction curve. Table 4.8 summarizes the proposed models' results and the obtained fitting coefficients at different extraction temperatures.

Table 4.8 : Summary of model fitting results based on linear coefficient, RMSE and X^2 at various temperatures

	T (°C)	R^2	RMSE ($x10^{-3}$)	X^2
First Rate Order	45	0.1879	2.2272	1.6818
	50	0.4881	3.2114	2.1130
	55	0.7992	3.6172	1.6814
	60	0.8110	2.9271	2.2011
	65	0.8514	2.1110	1.8114
Second Rate Order	45	0.9901	5.2548	0.3012
	50	0.9945	3.1228	0.2137
	55	0.9906	2.1028	0.0145
	60	0.9912	2.1338	0.1012
	65	0.9921	4.1228	2.2411
Peleg	45	0.9321	0.0231	0.1007
	50	0.9077	2.1228	0.5150
	55	0.9011	3.1228	1.3210
	60	0.8712	3.1228	1.1200
	65	0.8113	0.0014	0.0013

The highest R^2 and the lowest RMSE and X^2 were the criteria used to evaluate the model's suitability and represent the experimental data. The second-rate order was selected as it was found to correlate very well within the data analysis of all temperatures,with a higher and consistent linear correlation (R^2>0.99), and lower ranges of RSME andX^2 values.

Thus, this model is expected to be applicable to describe the extraction behavior of *o*-coumaric acid concentration using ultrasonic-assisted extraction method at various temperatures. Firstrate order rate order model failed to fit the experimental data and Peleg model was suggested as second option. The experimental results were further analysed using a second order model by plotting t/C_t versus time as shown in Figure 4.16.

Figure 4.16 implies a good agreement between the second order model and the experimental results with high coefficient of determination. This agreement confirmed theassumptions that ultrasonic assisted extraction of *G. sepium* leaf takes place in two subsequent stages of fast and slow.

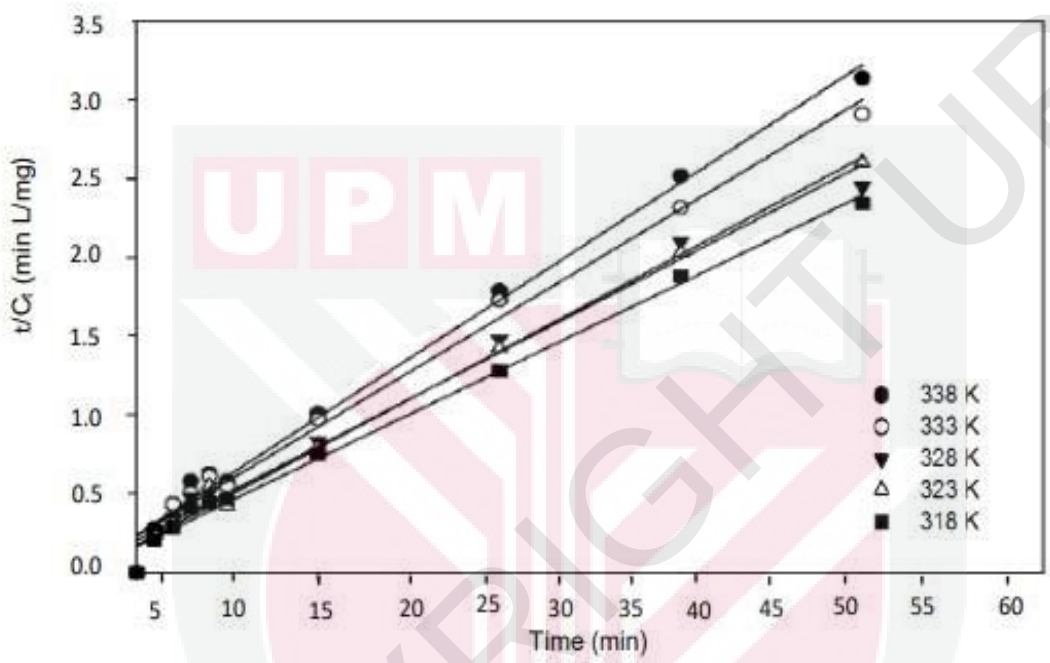


Figure 4.16 : Second order extraction kinetics of *G. sepium* leaf at different temperatures

From Table 4.9, all the saturated extraction capacity, C_s, extraction rate constant, k, and initial extraction rate, h, were linearly proportional to the extraction temperature. The C_s increases from 25.64 mg/L to 34.72 mg L^{-1} by increasing the temperature from 45 to 65°C.

For extraction constant rate, k, it increased from 4.75 x 10^{-3} to 7.67 x 10^{-3} L g^{-1} minand the initial extraction rate, h, increased from 3.12 to 9.24 mg L^{-1} min K with the increasetemperature from 45 to 65°C respectively.

Table 4.9 : Linearization of kinetic model of second order ultrasonic assisted extraction extraction of *G. sepium* leaf under different temperatures

T (°C)	Linear Equation	C_s (mg L^{-1})	k (L mg^{-1} min^{-1})	h (mg L^{-1} min^{-1})	R^2
45	y = 0.039x + 0.3204	25.64	4.75×10^{-3}	3.12	0.9901
50	y = 0.0373x + 0.2661	26.81	5.23×10^{-3}	3.75	0.9945
55	y = 0.0331x + 0.1801	30.15	6.11×10^{-3}	5.55	0.9906
60	y = 0.0308x + 0.1360	32.46	6.95×10^{-3}	7.32	0.9912
65	y = 0.0288x + 0.1081	34.72	7.27×10^{-3}	9.24	0.9921

4.6.3 Activation Energy

The second order extraction rate constants increase with the increasing temperatures aswas shown in Table 4.9. The phenomena can be described by the Arrhenius equation. Temperature independent factor (A) and activation energy of extraction (E) can further be calculated by plotting *ln* (k) against 1/T as can be seen in Figure 4.17.

From the graph below, there was a linear relationship between the second order extraction rate constant and the inverse temperature with linear equation of y = -2671.5x + 3.0406 and high R^2 value of 0.9942. Thus, *k* value;

$$k = 0.00757\mathrm{Exp}\left[\frac{-7309.67}{8.314\mathrm{T}}\right] \qquad (4.4)$$

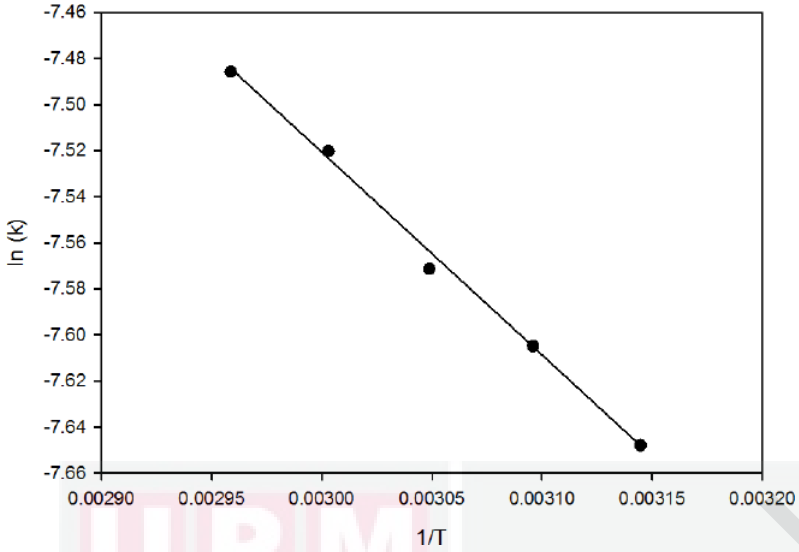


Figure 4.17 : Linear relationship between second order extraction constant, (*ln k*)and temperature for solid liquid extraction of *Gliricidia sepium* leaf

The temperature independent factor (A) and activation energy (E) were found to be 0.00757 L g^{-1} min and 7309.67 J mol^{-1} respectively from the slope and intercept. Since the activation energy is positive, thus the extraction of *G. sepium* leaf was considered toundergo endothermix reaction.

4.6.4 Extraction Process of Second Order Rate Model

A relationship between the saturated extraction capacity and temperature can beestablished by plotting C_s versus T as shown in Figure 4.18.

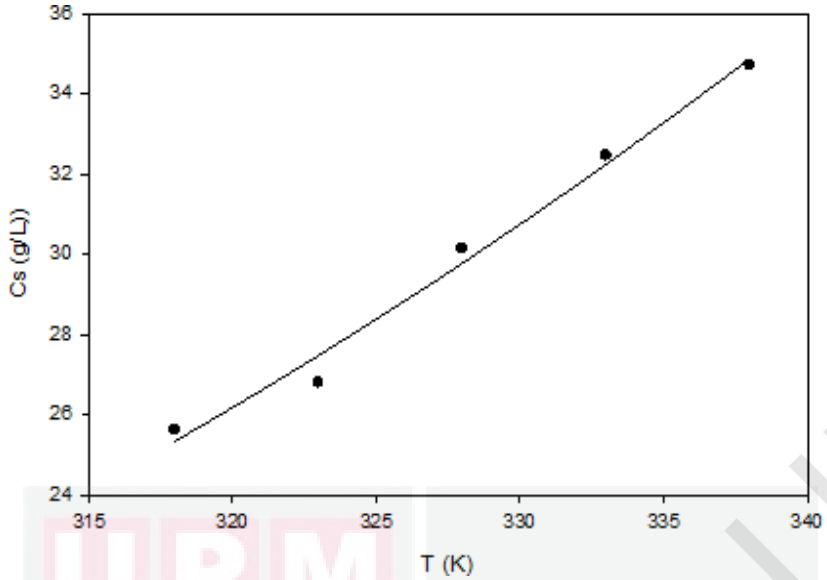


Figure 4.18 : Relationship between saturated extraction capacity, Cs, and temperature for ultrasonic-assisted extraction of *G. sepium* leaf

Polynomial equation was derived from the graph as Equation 4.5;

$$Y = 0.033X^2 - 1.679X + 227.09 \tag{4.5}$$

Therefore, C_s can be furthered correlated by the following Equation 4.6;

$$C_s = 0.0033T^2 - 1.6792T + 227.09 \tag{4.6}$$

Based on the relation between curve plot and Equation 4.6 by using experimental data, it showed that there was a positive correlation between saturated extraction capacity, $C_s$and temperature. C_s values are non-linearly proportional with extraction temperature. The values of *h* could also be obtained through the following Equation 4.7;

$$h = 0.00757\text{Exp}\left[\frac{-7309.67}{8.314\text{T}}\right]x(0.0033\text{T}2-1.6792\text{T}+227.09) \tag{4.7}$$

The evolution of C_t versus time and temperature for ultrasonic-assisted extractionextraction can be further described as;

$$C_t = \frac{t}{\frac{1}{h} + \left[\frac{t}{0.0033T2 - 1.6792T + 227.09}\right]} \quad (4.8)$$

Equation 4.8 represents the predictive model for ultrasonic-assisted extraction of *G. sepium* leaf using methanol for specific range of extraction time and temperatures. However, this equation is valid for only to predict the extracted amount of *o*-coumaric acid at any given extraction time up to 40 minutes and temperature in range of 45 to 65°C (318 to 338K). This finding indicated that more *o*-coumaric acid could be extracted at longer extraction temperature up to methanol boiling point.

By using the predictive model, the amount of *o*-coumaric acid (C_t) in different combination of time (t)and temperature (T) can be calculated. This feature is useful for economic assessment in industrial application. The kinetics of extraction was developed based on the assumption of a second order rateof extraction model with regression coefficient value of $R^2 > 0.99$. The *o*-coumaric acid concentrationobtained (Ct) was compared with the experimental results for the second order model validation at the lowest and highest temperature range limits. The good fitting was obtained for the model, showing a good relationship between experimental and calculated data.

4.7 Characterization of *O*-Coumaric Acid from *Gliricidia sepium* Leaf Extracts

The leaf structure of *Gliricidia sepium* was analysed before and after the drying and extraction process using several methods covering the size, shape and morphology, chemical and physichohemical characteristics.

4.7.1 Identification of *O*-Coumaric Acid Using High Performance LiquidChromatography

The extraction of *o*-coumaric acid from *G. sepium* leaves was conducted using optimized parameters. Several mobile phase combinations were tried and methanol: 0.4% acetic acid was found to be optimum for separation of *o*-coumaric acid from methanolic extract of *G. sepium* leaf. An accurate isocratic RP-HPLC method was developed and validated by optimised chromatographic conditions.

The obtained chromatograms showed a peak of *o*-coumaric acid as shown in Figure 4.19 (B). The regression coefficient obtained from linearity plot for *o*-coumaric acid was found as 0.9979, which indicates this method had good linearity. There was a major peak detected in dried leaves at wavelength 290nm and it was identified as *o*-coumaric acid by comparing their retention times at about 2.78 minutes. The chromatogram shows the similarity peak detected in *G. sepium* leaf extractas comparable to the standard, indicating the presence of *o*-coumaric acid and the compound identification was successfully achieved.

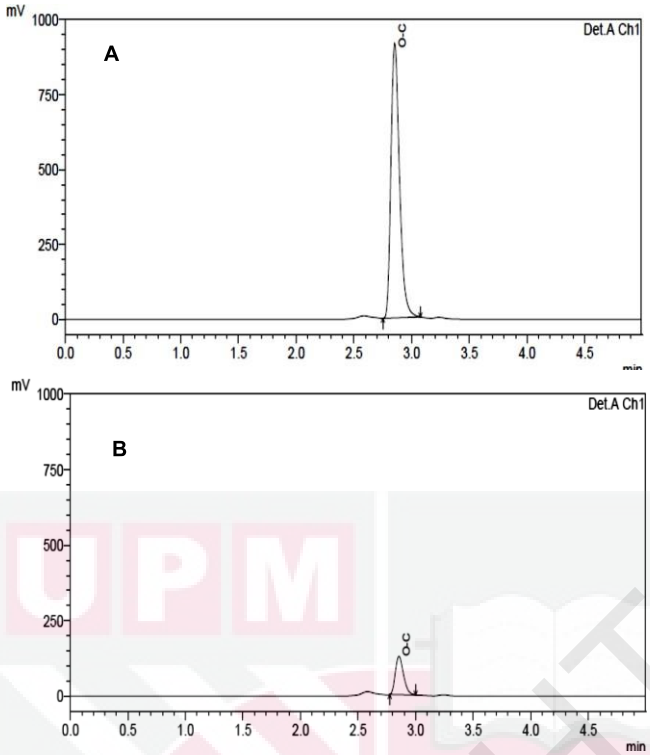


Figure 4.19 : HPLC chromatograms of *o*-coumaric acid (A) standard; (B) dried extract at retention time of 2.78 min

4.7.2 Characterisation of *O*-Coumaric Acid Using Fourier-Transform InfraredSpectroscopy

The determination and identification of the functional groups of *o*-coumaric acid were then confirmedthrough the infrared spectroscopy by superimposing its spectra with the standard. Basedon Figure 4.20, *o*-coumaric acid structure ($C_9H_8O_3$) was dispersed by the FT-IR spectra on the following grounds as summarized in Table 4.24.

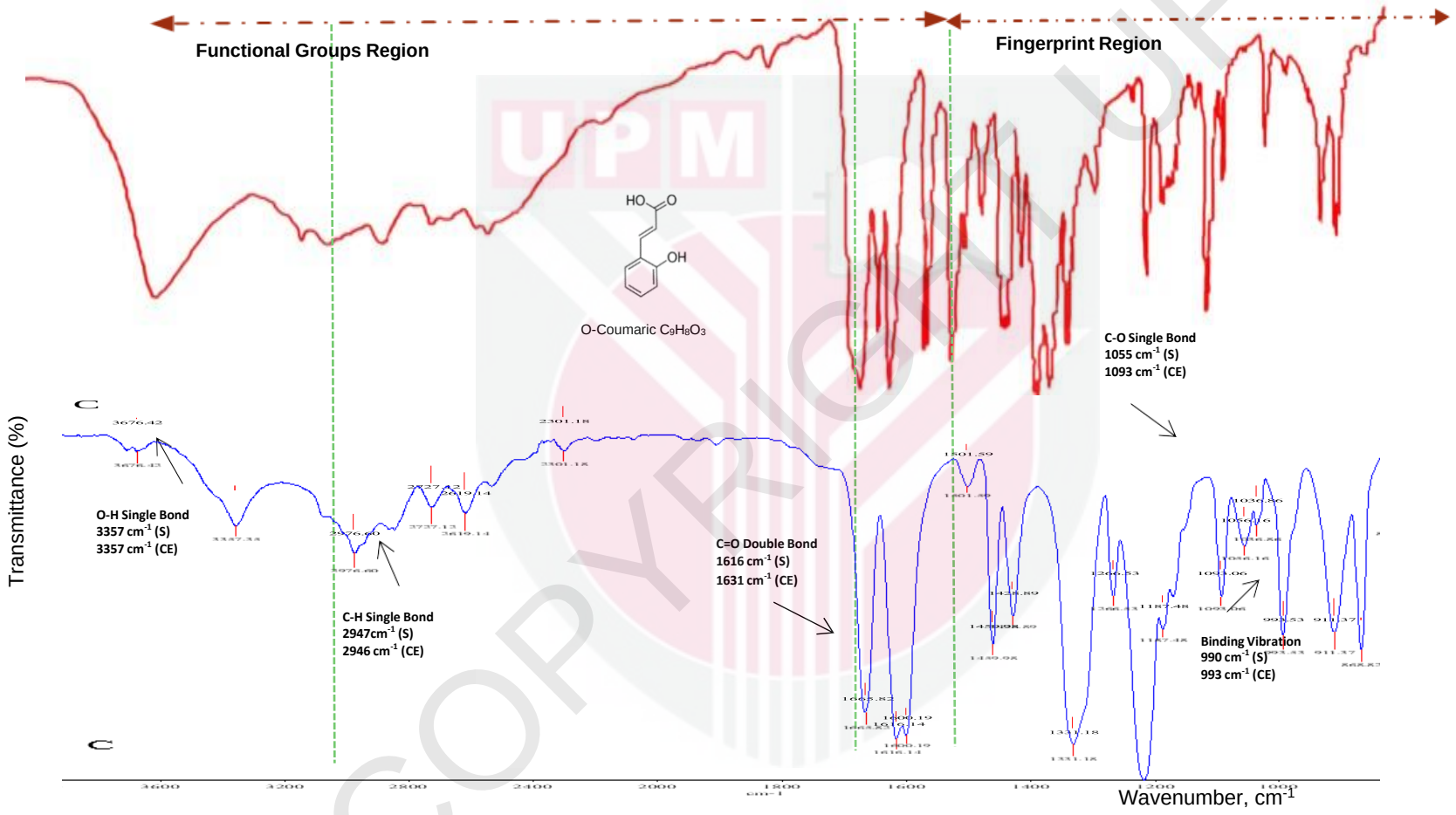


Figure 4.20 : Experimental FTIR spectra of *o*-coumaric acid comparing standard (S) in red lines with crude extract (CE) in blue lines

Table 4.10 : FTIR spectra of *O*-Coumaric Acid

Chemical Bonds	Stretching Frequencies
O-H single bonds	Based on the IR spectra of the *o*-coumaric acid standard, there is a slightly broad and intense –OH single bond stretching frequencies at 3354 cm^{-1} (S)as compared to 3357 cm^{-1} (CE).
C-H single bonds	At the region of C-H stretching frequencies, there are two or three bands of weak to medium intensity have been observed at the regionof 2945 (S), to 2976 cm^{-1} (CE). Bands are located in the range 2045- 2945 (S), 2301-2936 cm^{-1} (CE), can be assigned to the C-H stretching modes of aromatic ring and the double bond between C_8 and C_9 atoms.
C=O double bonds	The C=O stretching frequency of *o*-coumaric acid is usually observed at theregion 1700 to 1750 cm^{-1}. The IR spectra of *o*-coumaric acid showed as strong C=O absorption at 1616 (CE) to 1631 cm^{-1}(S).
C=C skeletal vibrations	A peak around 1616 cm^{-1} (S), 1631 cm^{-1} (CE), derives from the C-C stretching vibrations of the double bond.
Other absorptions	The bands at the range of below 1000 cm^{-1} with 990 cm^{-1} (S) and 993 cm^{-1} (CE) are assigned to out-of-plane bending vibrations whilethe bands with 1055 cm^{-1} (IR) and 1097 cm^{-1} (experimental) are C-C single bonds.

4.8 Chapter Summary

The overall conclusion was made based on the whole drying and extraction process as below;

1. The effect of oven drying temperature and time on the quality and drying kinetics of *G. sepium* leaves was successfully studied. All the drying operation temperatures occur in the falling rate period and short constant rate period in the middle of drying process. The drying kinetics of G. *sepium* leaves were successfully modelled using Midilli-Kucuk model.

2. The quality of bioactive compound from the dried *G. sepium* leaves was successfully identified as the required compound, *o*-coumaric acid with optimum drying temperature of 50°C.

3. For the extraction kinetic parameters, namely initial extraction rate (h), the extractionrate constant (k), the concentration of saturation (C_s) and the activation energy (E_a) were successfully calculated using

the obtained model. The value of activation energyas E_a=7309.67 J mol^{-1} indicated the endothermic nature of the UAE process.

4. The best optimization conditions using the UAE method lies on extraction temperature of 47°C, extraction time of 45 min and R_{ss} of 10:1 mg L^{-1} resulted to *o*-coumaric acid concentration of 36.14 ±0.015 mgL^{-1}.

5. The desired extracted compound of *o*-coumaric acid was successfully identified through HPLC at retention time of 2.78 minutes and its functional groups were confirmed through FTIRanalysis, indicating the extracted compound of *o*-coumaric acid from G. *sepium* was identical to the commercial *o*-coumaric acid.

CHAPTER 5

FERMENTATION PROCESS BY *ASPERGILLUS FUMIGATUS FRESENIUS* NRRL 163 FOR BIOCONVERSION OF O-COUMARIC INTO DICOUMAROL, GROWTH KINETICS AND COMPOUND CHARACTERISATION

5.1 Introduction

Bioconversion of dicoumarol is an alternative process among other green technologies to enhance the rodenticide anticoagulant activity which is efficient and cost effective. However, it requires an overall optimization to become costly competitive. *Aspergilli* are the best dicoumarol producer especially for the species of *Aspergillus fumigatus Fresenius* (Bye and King, 1970). In this research, fermentation process is used for bioconversion of converting *o*-coumaric acid extracted from the *G. sepium* leaf into dicoumarol. Thefermentation process was carried out using shake flask to enhance the activity of dicoumarol as rodenticides anticoagulant. The process was enhanced through the biological approach using *A. fumigatus* NRRL 163 followed the method used by Bye andKing (1970) with some modifications.

The fermentation yield in shake flask system is strongly influenced by several culture conditions. In this study, the optimization was carried out using one factor at one time todetermine the effect of media composition, initial glucose and nitrogen concentration as among the most significant factors that influence the *A. fumigatus* growth based on singleexperiment that has been carried out. A mathematical correlation between the parameters were developed to obtain an optimum of dicoumarol production. The responses were dicoumarol yield, maximum dicoumarol concentration and volumetric productivity.

Microorganisms grow in a wide spectrum of physical and chemical environments, where their growth rate are closely influenced by their physiological activities. Fermentation kinetic describes growth and product formation not only during active cell growth but also during the activities of resting and dying cells. The objective of the study was to analyze experimental data of batch fermentation of *A. fumigatus* NRRL 163 producing dicoumarolfor generation of kinetic parameter values that are subsequently used to form the basis kinetic study of the process.

5.2 Shake Flask Fermentation Process for Bioconversion of *O*-Coumaric Acid into Dicoumarol

5.2.1 Strain Propagation of Aspergillus fumigatus Fresenius NRRL 163

The strain of *A. fumigatus* NRRL 163 from a lyophilised ampoule was well propagated indefined medium (potato dextrose broth) and basal salt (complex medium) after overnight incubation. The whitish mycelium turned into olive green and velutinous to cotton colonies after 22 incubation days on the basal salt agar at room temperature of 25°C. The conidia were verrucous and olive green in colour as shown in Figure 5.1.

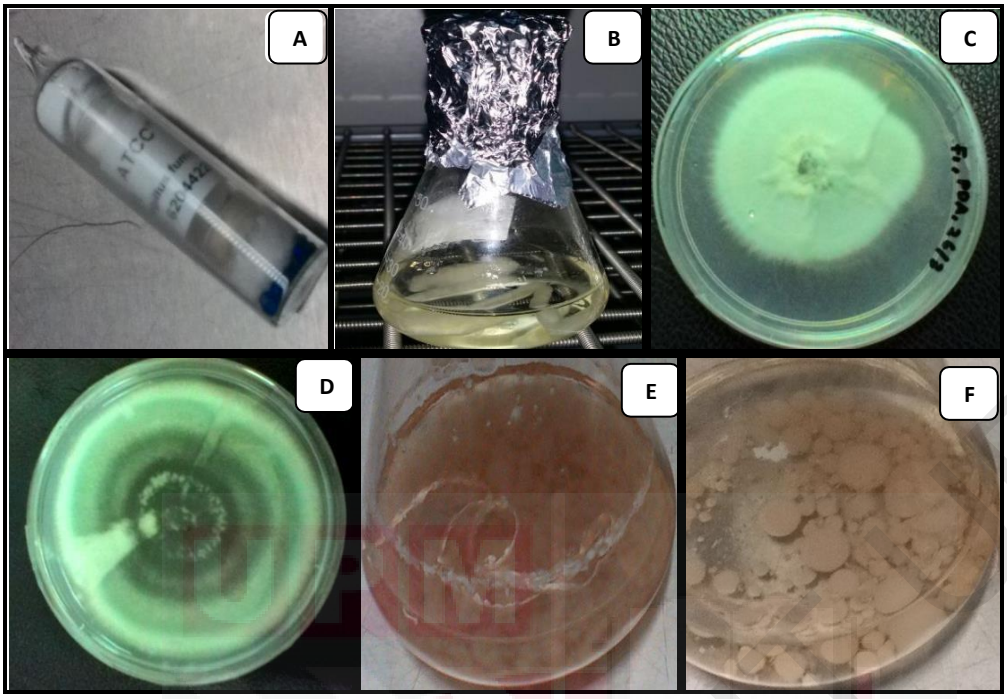


Figure 5.1 : The strain of *A. fumigatus* NRRL 163; (A) ATCC® 1022™ strain in a lyophilised ampoule, (B) master culture stock in PDB, (C, D) strains propagation on PDA plates, (E, F) cell cultures in defined and complex medium

5.2.2 Solid State Fermentation

The fermentation process was initiated with solid state fermentation to enhance the microbe incubation on solid surfaces for its optimum growth. The fresh and grounded leaves *G. sepium* leaves were sterilized using surface sterilization method as suggested by Daud *et al.* (2013) with some modifications. The inoculum growth on the substrate wasobserved after three incubation days with dense mass of whitish to light greenish coloniesspread over the leaf surfaces as shown in Figure 5.2.

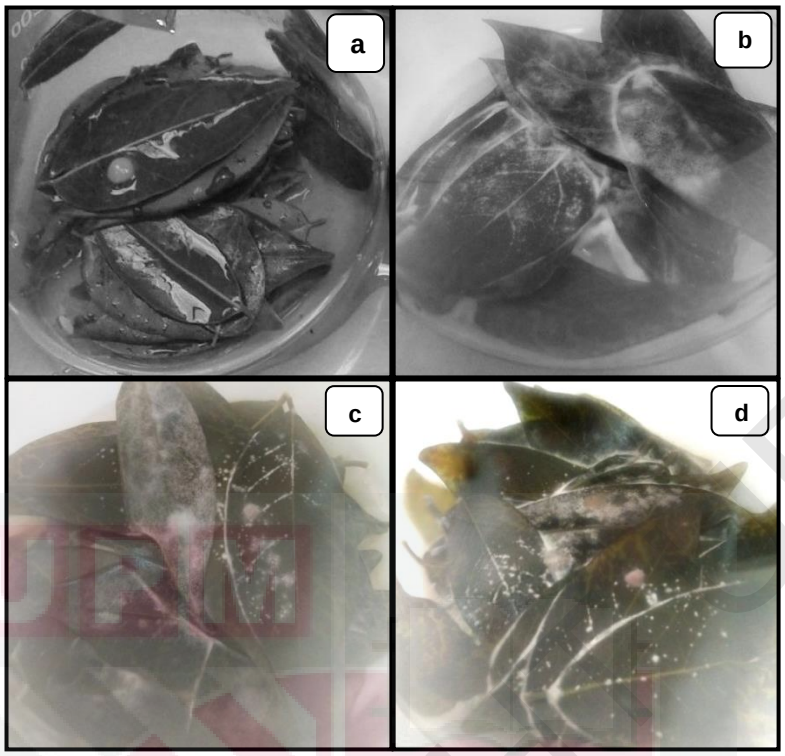


Figure 5.2 : Inoculum on substrate (a), strains growth after (b) 3 days, (c) 5days and (d) 7 days of incubation periods

The inoculum growth was triggered by the extract of *o*-coumaric acid as carbon sources added with the glucose produced the thick surface layer of *A. fumigatus* NRRL 163 on the *G. sepium*leaves. The existing *o*-coumaric acid as carbon sources from dried leaves added with dried extract powder could enhance the cell growth as second substrate after glucose. The carbon content was about 10% as been proven by the Proximate Analysis in Section 4.6.4.

The whitish colonies have been initially observed after three incubation days as shown in Figure 5.2 (b) and colonies started to growth up to 7 days (Figure 5.2c-d) and the colonies growth has stopped and no increase in mass production has been observed after 7 daysof incubation period indicating the

end of growth curve due to exhausting carbon sources.

5.3 Optimization of Submerged Fermentation using One Factor at One Time

5.3.1 Effect of Complex and Defined Media on the Dicoumarol Production

Initially, the feasibility of two different media on growth of *A. fumigatus* NRRL 163 and dicoumarol production was investigated. Submerged fermentation was conducted in 500 mL flasks with 200mL working volume of complex medium potato dextrose containing 4 gL^{-1} potatoes extract, 20 L^{-1} dextrose and 20 L^{-1} agars.

About 10% of inoculum size containing minimum of 1.0×10^6 spores/mL were inoculated into each flaskand flasks were shaken at 250 rpm at 30 °C. Regular sampling was done at defined time intervals for viability test and biomass determination. The fermentation process was repeated using defined medium containing 5 g L^{-1} D-glucose, $NaNO_3$ (6g), KCl (0.52g), $MgSO_4.7H_2O$ (0.59g), KH_2PO_4 (1.52g) and trace elements of $FeSO_4.7H_2O$ (4mg), $ZnSO_4.7H_2O$ (0.4mg).

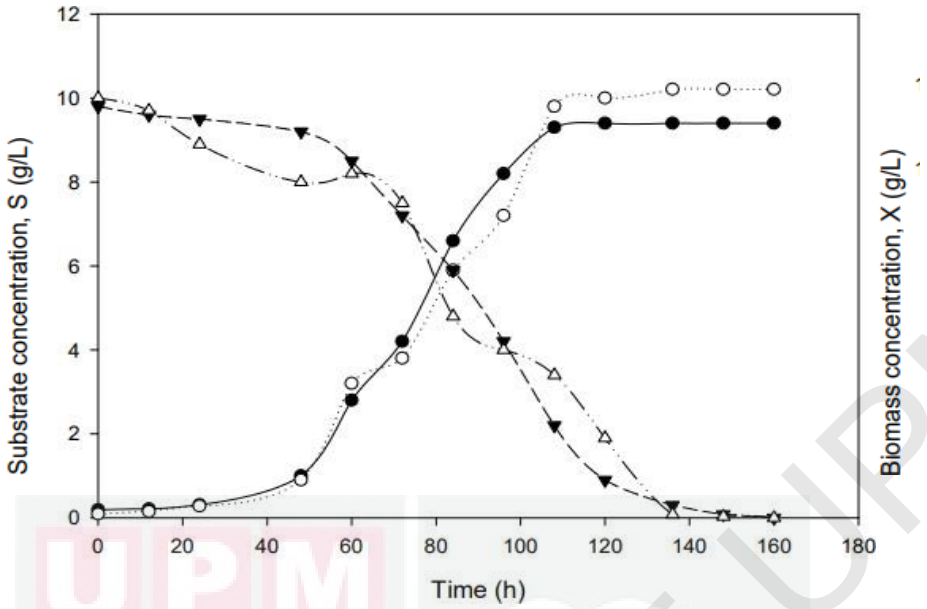


Figure 5.3 : Time course of batch cultivation of *A. fumigatus* in shake flask using D-glucose as a carbon source in defined medium (●) substrate; (▼) biomass and complex medium (○) substrate; (▽) biomass

The kinetics constant, μ_{max} and kinetics parameter, K_s were determined on the curve fitting method as shown in Figure 5.4. The values of specific growth rate, μ was calculated according to the cell dry weight of fungal biomass concentration, X and glucose concentration as limiting substrate, S during the exponential growth phase. Based on the Figure 5.4, the maximum specific growth rate, μ_{max};

$$\mu_{max} = \frac{\ln 4.2 - \ln 0.9}{84-72}$$

$$\mu_{max} = 0.12\ h^{-1}$$

$$\text{The yield on susbstrate, } Y_{x/s} = \frac{-\ 9.3\ \text{g cells}\ L^{-1}}{(0\text{-}9.8)\ \text{substrate}\ L^{-1}}$$

$$= 0.95\ \text{g cells g substrate}^{-1}$$

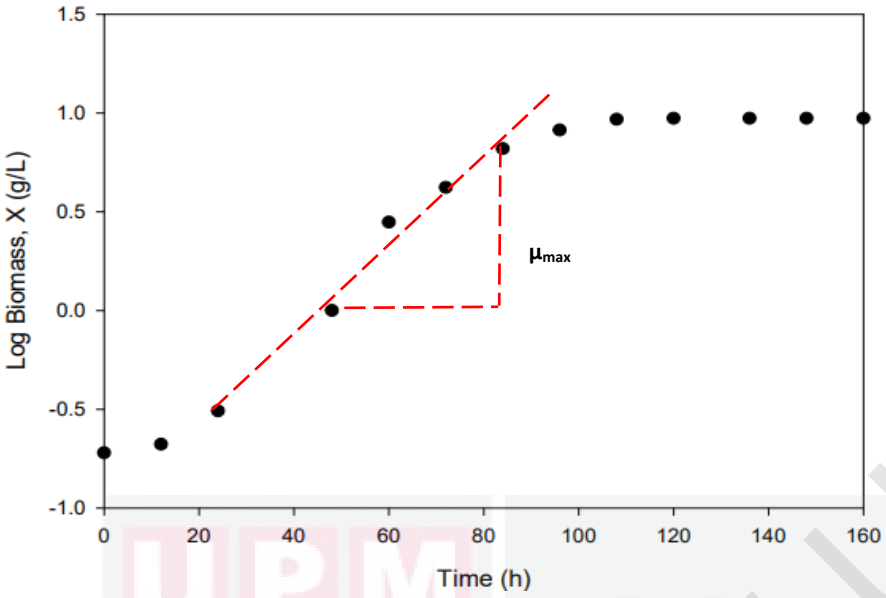


Figure 5.4 : Time course of log biomass for U_{max} determination

For monod saturation constant, k_s the graph of specific growth rate, μ vs subsrate (s) was determined from Lineweaver Burk Plot as in Figure 5.5.

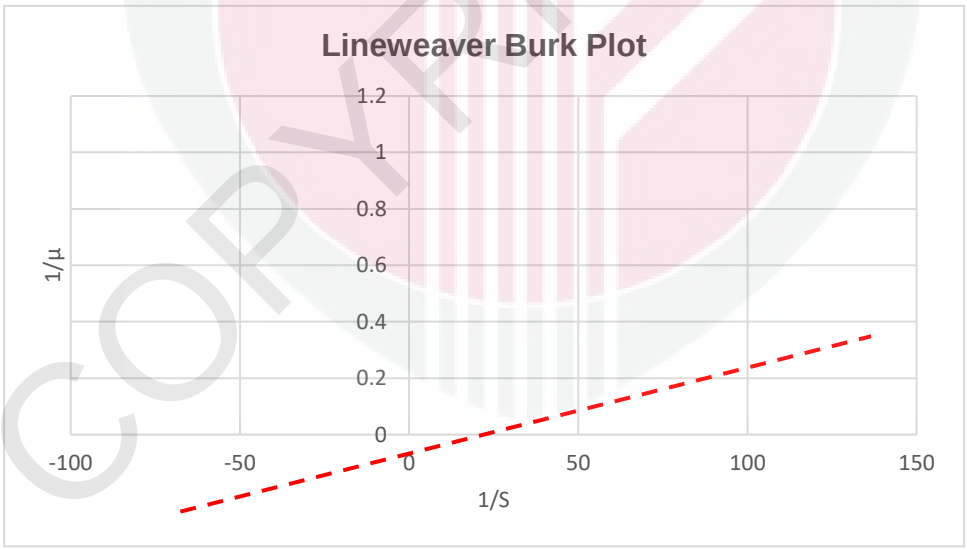


Figure 5.5 : Lineweaver Burk Plot for determination of monod saturation constant

From the plot, the slope therefore,

$$k_S = 0.26 \times 0.12 h^{-1} = 0.0312 \text{ g cells substrate } L^{-1}.$$

The specific growth rate, μ_{max}, was measured using the slope of ln x vs time at the log phase of the growth curve for each of the culture in both medium. The μ_{max} in complex medium is about 45% higher compared to growth in defined medium. This showed that the higher μ_{max} will decrease the tendency for the dicoumarol production by *A. fumigatus* NRRL 163. Biomass concentration was determined by measuring biomass dry weight per liter sample after 7 days of incubation period.

Although higher final biomass concentration (9.33 g L^{-1}) was obtained in fermentation using complex medium (8.61 gL^{-1}), dicoumarol production (0.214 U L^{-1}) was significantly reduced. High dicoumarol production (0.332 U L^{-1}) obtained in fermentation using defined medium indicated that the presence of simple sugars and compounds that can be utilized easily bythe *Aspergillus* enhanced the cell built-up with higher ability to trigger dicoumarol production.

The measure of cell efficiency (P_{max}/X_{max}), calculated as dicoumarol activity per unit cell weight (0.035 U g $cell^{-1}$) was about 40% higher for fermentation using a defined medium as compared to complex medium (0.021 U g $cell^{-1}$). The product yield ($Y_{p/s}$)in defined medium was higher compared to complex medium showed that dicoumarol is more favourable produced in defined medium compared to complex medium. The comparison of kinetic parameters data was shown in Table 5.1.

Table 5.1 : Comparison of the performance and the kinetic parameter values of dicoumarol production in shake flask by *A. fumigatus* NRRL 163 using two different types of basal medium

Kinetic Parameter Values	Types of Basal Medium	
	Complex Medium (Potato Dextrose, PD)	**Defined Medium (Bye and King, 1970)**
Maximum specific growth rate, μ_{max} (h^{-1})	0.25	0.12
Maximum biomass concentration, X_{max} (g L^{-1})	10.21	9.33
Maximum dicoumarol concentration, P_{max} (U L^{-1})	0.214	0.322
Cell efficiency, P_{max}/X_{max} (U g $cells^{-1}$)	0.021	0.035
Cell yield, $Y_{x/s}$ (g cells glucose $^{-1}$)	1.18	0.95
Overall biomass productivity, P_x (g L·h^{-1})	0.13	0.12
Time to reach maximum dicoumarol production, t (h)	110	110

5.3.2 Effect of Initial Glucose Concentration on Dicoumarol Production

The performance of cell concentration, glucose and dicoumarol concentration during fermentation of *A. fumigatus* NRRL 163 using different concentrations of glucose was shown in Figure 5.6 with kinetic parameter values as shown in Table 5.2. The maximum cell concentration (X_{max}) increased with increasing glucose concentration from 0 g L^{-1} to 25 g L^{-1}, though the maximum specific growth rate (μ_{max}) was not significantly different.

However, the highest dicoumarol production (0.349 U L^{-1}) was obtained at 10 g L^{-1} glucose. Dicoumarol production started to reduce with higher glucose concentration more than 15 g L^{-1}. The highest overall dicoumarol productivity (153.81 U mL h^{-1}), P_{max}/X_{max} (0.027 g L $cell^{-1}$) and overall dicoumarol productivity (4.15 U mL·h^{-1}) were obtained at 10 g L^{-1} glucose.

The highest overall dicoumarol productivity corresponded well to the highest efficiency of the cell in producing dicoumarol. The product was formed simultaneously with growth of cells and the product concentration increases with cell concentration.

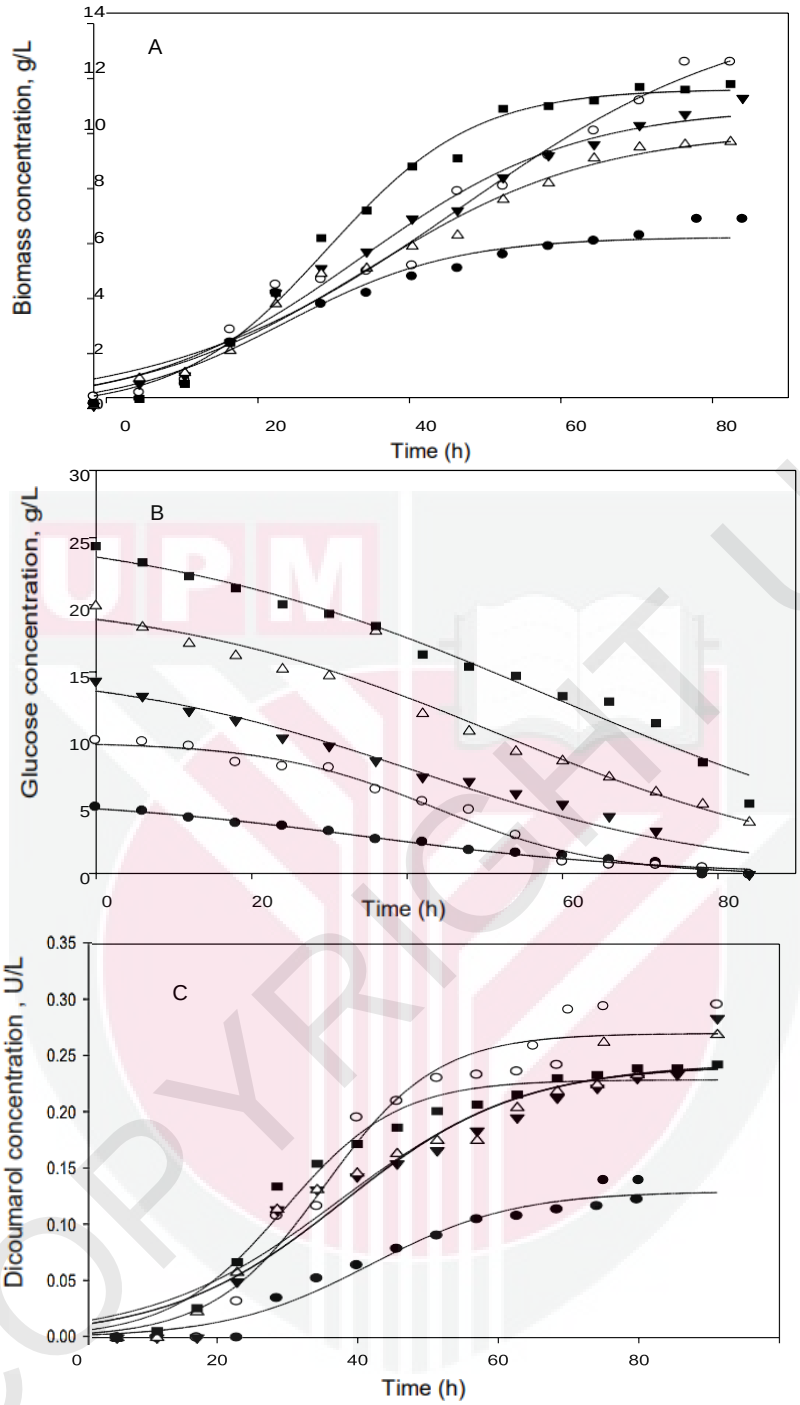


Figure 5.6 : Effect of different initial glucose concentrations on dicoumarol production by *A. fumigatus* NRRL 163 in defined medium in shake flask culture. (A)Cell concentration; (B) Glucose concentration; (C) Dicoumarol concentration. ●– 5 g L^{-1}; ○ -10 g L^{-1}; ▼ – 15 g L^{-1}; ∆ - 20 g L^{-1}; ■ - 25 g L^{-1}

Table 5.2 : Comparison of performance and kinetic parameter values of dicoumarol production in submerged culture system of *Aspergillus fumigatus Fresenius* using defined medium with different concentrations of glucose in shake flask fermentation

Kinetic Parameters	Initial Glucose Concentration (g L^{-1})					
	0	**5**	**10**	**15**	**20**	**25**
Maximum specific growth rate, μ_{max} (h^{-1})	0.02^{a}	0.29^{a}	0.49^{b}	0.45^{b}	0.44^{b}	0.12^{a}
Maximum biomass concentration, X_{max} (g L^{-1})	4.32^{a}	6.51^{b}	12.92^{e}	9.90^{d}	9.71^{d}	10.80^{c}
Maximum dicoumarol concentration, P_{max} (U L^{-1})	0.02^{a}	0.10^{b}	0.349	0.285^{d}	0.231^{d}	0.221^{c}
Final glucose concentration (g L^{-1})	0	0.1	0.2	4.9	7.86	9.25
Amount of glucose consumed (g L^{-1})	0	4.9	9.8	10.1	12.14	15.75
Cell efficiency, P_{max}/X_{max} (g L $cell^{-1}$)	0.004^{a}	0.015^{b}	0.027^{c}	0.028^{c}	0.024^{c}	0.019^{b}
Overall biomass productivity, P_x (g mL·h^{-1})	51.43^{a}	90.42^{b}	153.81^{e}	117.86^{c}	134.86^{d}	150^{e}
Overall dicoumarol productivity, $P_{dicoumarol}$ (U mL·h^{-1})	1.42^{a}	3.9^{b}	4.15^{c}	3.39^{b}	3.21^{b}	3.07^{b}
Time to reach maximum biomass, t (h)	84	72	84	84	72	72

[a,b and c]: Means values with the same letter are not significantly different

5.3.3 Effect of Initial Nitrogen Concentration on Dicoumarol Production

The profiles of cells concentration and dicoumarol activity using different concentrations of $NaNO_3$ are shown in Figure 5.7 while Table 5.3 shows the summary of fermentation performance and the kinetic parameter values. The maximum cell concentration (X_{max}) increased significantly with increasing $NaNO_3$ concentration up to 5 g L^{-1}. A slight inhibitionof growth was observed at 0 g L^{-1} $NaNO_3$. However, the highest maximum dicoumarol concentration (0.38 U L^{-1}) was obtained at 2 g L^{-1} of $NaNO_3$. Dicoumarol production was inhibited at $NaNO_3$ concentration higher than 3 g L^{-1}.

The maximum specific growth rate (μ_{max}) did not vary significantly with increasing $NaNO_3$concentration from 0 g L^{-1} to 2 g L^{-1}. On the other hand, the cell efficiency (P_{max}/X_{max}) in producing dicoumarol reduced with increasing $NaNO_3$ concentration, suggesting that ammonium gave inhibition effect to dicoumarol production by *A. fumigatus* NRRL 163. Thisis in agreement with Quiterio G. *et al* (2023), that claims high concentration of the carbonsource and low availability of the nitrogen source are key features to enhance growth of *Aspergillus* sp.

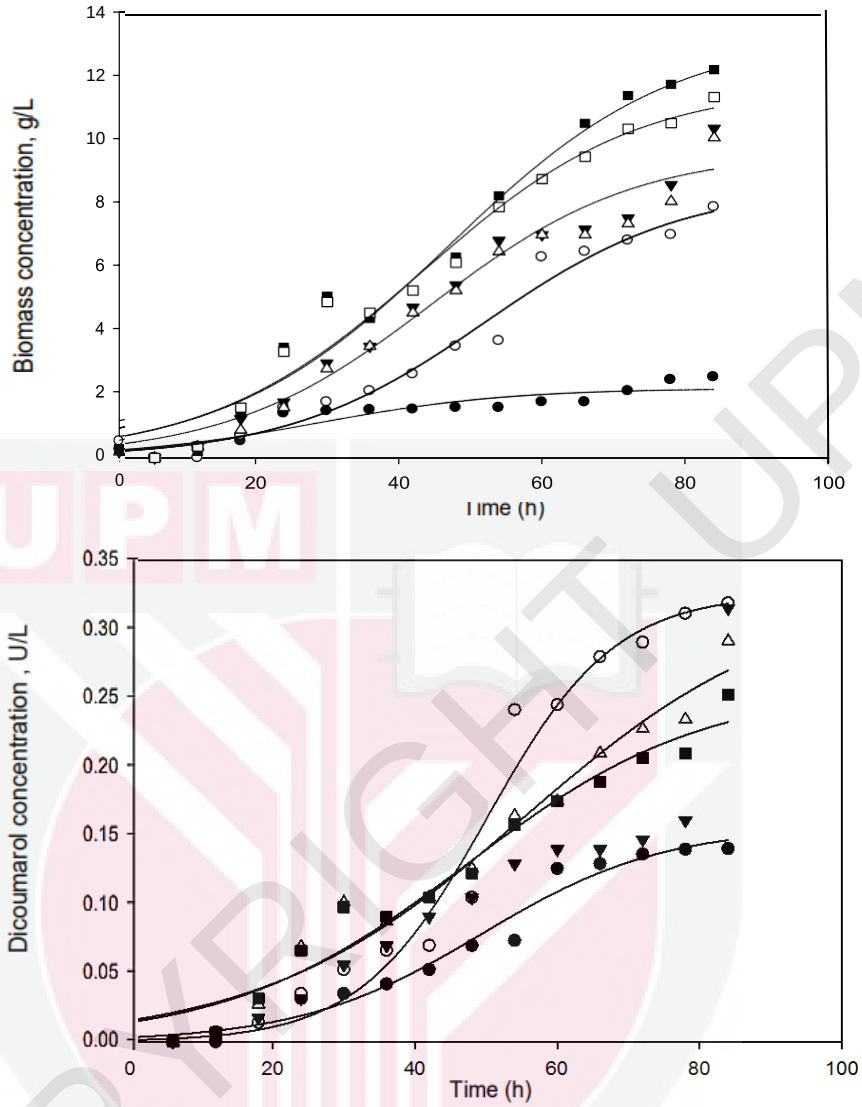


Figure 5.7 : Effect of different initial nitrogen concentrations on dicoumarol production by *A. fumigatus* in defined medium in shake flask culture. (A) Cell concentration; (B) Dicoumarol concentration. ●– 1 g L^{-1}; ○ -2 g L^{-1}; ▼ – 3g L^{-1}; ∆ - 4g L^{-1}; ■ - 5 g L^{-1}

Table 5.3 : Comparison of performance and kinetic parameter values of dicoumarol production in submerged culture system of *Aspergillus fumigatus Fresenius* using defined medium with different concentrations of $NaNO_3$ in shake flask fermentation

Kinetic Parameters	Nitrogen Concentration (g L^{-1})					
	0	**1**	**2**	**3**	**4**	**5**
Maximum specific growth rate, μ_{max} (h^{-1})	0.04	0.05	0.10	0.15	0.12	0.11
Maximum biomass concentration, X_{max} (g L^{-1})	0.45^{a}	4.56^{b}	8.91^{e}	9.04^{d}	10.26^{c}	11.11^{c}
Maximum dicoumarol concentration, P_{max} (U L^{-1})	0.04^{a}	0.20^{b}	0.38^{d}	0.24^{c}	0.29^{b}	0.24^{b}
Cell efficiency, P_{max}/X_{max} (g L $cells^{-1}$)	0.089^{d}	0.044^{c}	0.043^{b}	0.027^{b}	0.022^{a}	0.026^{a}
Overall biomass productivity, P_x (g mL·h^{-1})	6.25^{a}	63.33^{b}	106.07^{c}	107.6^{c}	122.1^{d}	132.26^{d}
Overall dicoumarol productivity, $P_{dicoumarol}$ (U mL·h^{-1})	0.56^{a}	2.78^{b}	4.52^{d}	2.85^{b}	2.74^{b}	3.45^{c}
Final nitrogen concentration (g L^{-1})	0	0	0	0	0.91	1.12
Amount of nitrogen consumed (g L^{-1})	0	1	2	3	3.09	3.88
Time to reach maximum dicoumarol production, t (h)	72	72	84	84	84	84

[a,b and c]: Means values with the same letter are not significantly different

5.4 Growth Kinetics of *Aspergillus fumigatus Fresenius* NRRL 163 forDicoumarol Production

The cell growth kinetics were evaluated as a function of cell dry weight versus time. Initially, the submerged fermentation was carried out with the optimal defined media consisted of 5 g L^{-1} leaf extract, 5 g L^{-1} D-glucose, $NaNO_3$ (6g), KCl (0.52g), $MgSO_4.7H_2O$ (0.59g), KH_2PO_4 (1.52g) and trace elements of $FeSO_4.7H_2O$ (4mg), $ZnSO_4.7H_2O$ (0.4mg), 10 g L^{-1} glucose and 2 L^{-1} of $NaNO_3$ using shake flasks fermentation system. The fermented media consisted of 200mL working volume and 10% inoculum size was incubated on orbital shaker at 30 °C and agitated at 250 rpm. The culture pH was initially maintained at around 7 throughout the fermentation process.

In shake flasks fermentation, the growth of *A. fumigatus* NRRL 163 was very rapid, as short lag phase was observed and the growth reached stationary phase after about 80 h. Duringrapid growth, glucose was consumed at a very high rate and glucose depletion was observed before the growth ends the stationary phase as shown in Figure 5.8. In term of energy source, dicoumarol production started to reduce with higher glucose concentration more than 15 g L^{-1} indicating excessive carbon sources will deplete the bioconversion process. Dicoumarol also reduced with increasing $NaNO_3$ concentration, suggesting that ammonium gave inhibition effect to dicoumarol production by *A. fumigatus* NRRL 163.

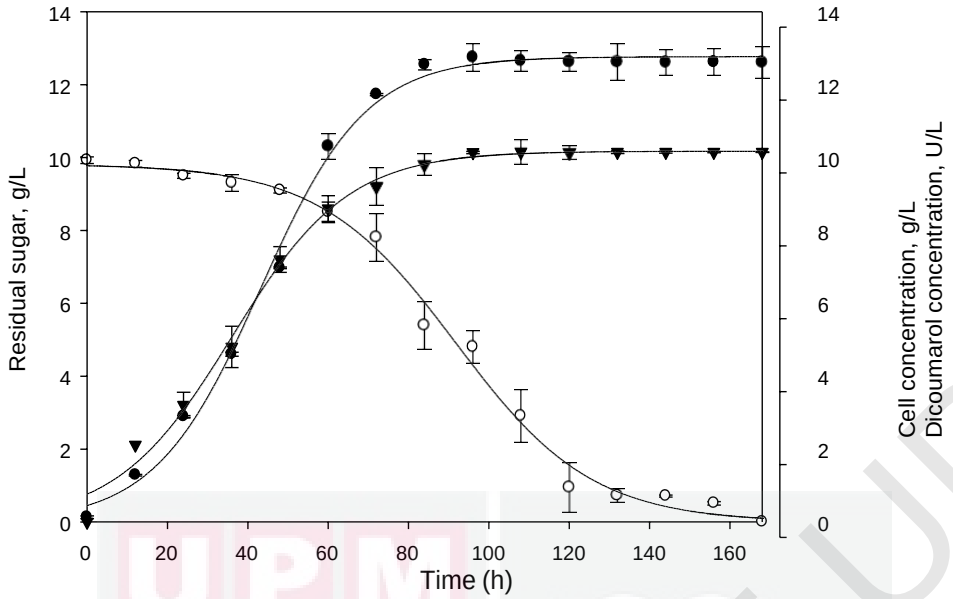


Figure 5.8 : Time course of batch cultivation of *A. fumigatus* in shake flask using glucose as a carbon source. (○) sugar; (●) biomass; and (▼) dicoumarol

In shake flasks fermentation, the growth of *A. fumigatus* NRRL 163 was very rapid, as nolag phase was observed and the growth reached stationary phase after about 16 h. Duringrapid growth, glucose was consumed at a very high rate and glucose depletion was observed before the growth reached stationary phase. Dicoumarol production was increased concomitantly with growth, demonstrating that the process is growth associated. The culture pH was initially maintained at around 7 throughout the fermentation process. From Figure 5.8, it is very clear that dicoumarol was produced concomitantly with the growth of *A. fumigatus*. During active growth phase, some of glucose and nitrogen were required to promote growth and enhancement of the biosynthesis of enzymes involved in dicoumarol production.

5.5 Identification and Characterisation of Dicoumarol Compound

5.5.1 Separation of Dicoumarol Using HPLC Fractionation

Reversed phase separation mode is most commonly used for HPLC separation of mixtures of coumarin derivatives including of dicoumarol as documented in Table 5.5. In this study, the work focused on development of HPLC method for separation and determination of dicoumarol.

Figure 5.9 shows the comparison of chemical profiles between standards and the sample fraction with identical major peak detected at UV detection wavelength of 303 nm. The major peak was identified as dicoumarol by comparing their retention time with the standards dicoumarol which were about 2.29 min. The chromatogram of both is slightly different in which the peak of dicoumarol sample fraction was smaller compared to the standard and was proven by HPLC chromatogram. There is also a peak detected at the retention time of 2.78 min identified as remaining *o*-coumaric acid.

The amounts of dicoumarol sample fraction were calculated from the peak area by using the calibration curves developed from the standards as the control in the analysis. The results indicate that the concentration of dicoumarol sample fraction was identical to the standard with the same retention time.

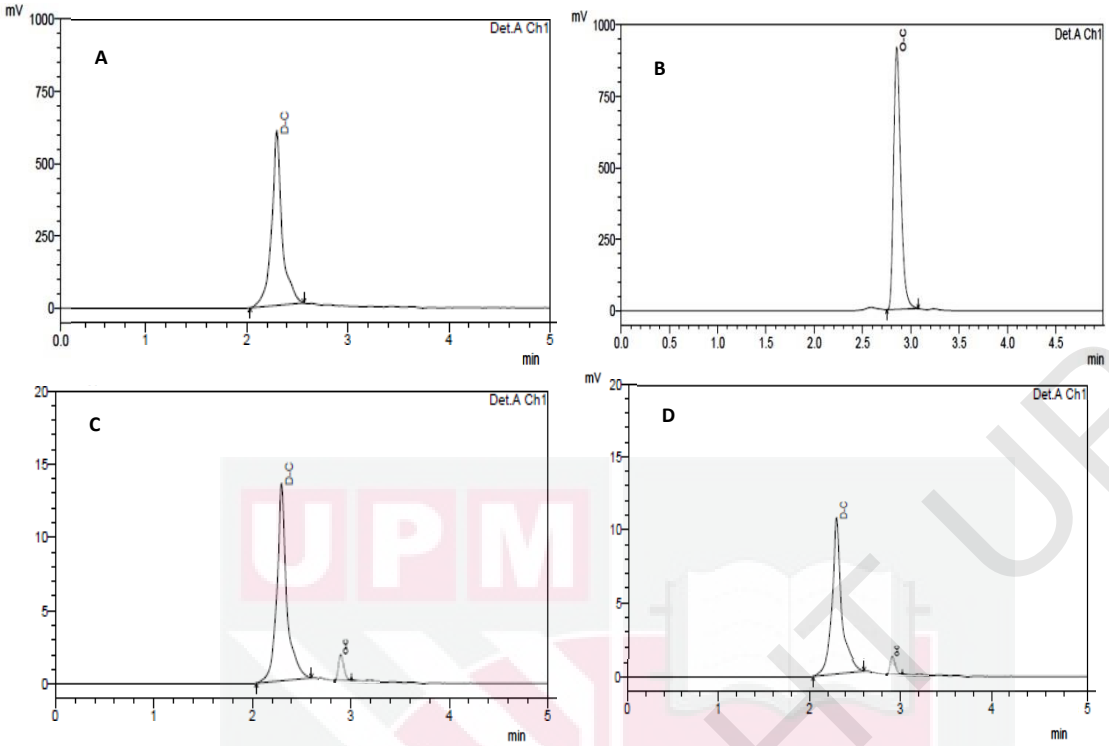


Figure 5.9 : Chromatograms of dicoumarol using HPLC fractionation method (A) dicoumarol standard; (B) *o*-coumaric acid standard; (C) sample fraction of dicoumarol in definedmedium and (D) sample fraction in complex medium with methanol-acetic acid mobile phase composition at 303 nm of wavelength

This result confirmed that the sample fraction was the desired dicoumarol compound with the recoveries of 90% subjected to the extraction and purification procedure for 60ppm solution. The similar result also has been reported by Hrobonova *et al.* (2018) in their studyon sweet clover with 88.6 to 92.6% of dicoumarol recovery subjected to HPLC-DAD analysis. This finding is supported by Alister and Bernard (1992) in their study of dicoumarol in *Sweetclover* hay and silage samples.

5.5.2 Characterisation of Dicoumarol Using Fourier-Transform InfraredSpectroscopy

The determination and identification of the functional groups of the converted sample of dicoumarol were then confirmed through the infrared spectroscopy by superimposing its spectra with the standard. Based on Figure 5.9, dicoumarol structure ($C_{19}H_{12}O_6$) was spread by the FT-IR spectra on the following grounds as summarized in Table 5.4;

Table 5.4 : FTIR Spectra of Dicoumarol

Chemical Bonds	Stretching Frequencies
O-H single bond	Based on the spectra of the dicoumarol standard, there is a broad and intense –OH single bond stretching frequencies at the range of 3000 to 3200 cm^{-1}.
C-H single bond	At the region of C-H stretching frequencies, there are two or three bands of weak to medium intensity have been observed at the region of 2618 to 2735 $cm^{-1.}$ These absorptions were due to the C-H stretching vibrations of the pyrene, benzene and furan rings.
C=O double bond	The C=O stretching frequency of dicoumarol is usually observed at the region 1700 to 1750 cm^{-1} whereas in chromones (γ-pyrones) it was found at ~1650 cm^{-1}. The 6-O and 7-O- coumarin glycosides have their C=O frequencies below 1700cm^{-1}. Infrared spectrum reveals absorption bands around 1737 cm^{-1} which was assigned to the C=O stretching frequency of the ketone. The standard spectra of dicoumarol showed as strong C=O absorption at 1660 to 1680 cm^{-1}. This was presumably due to the intramolecular hydrogen bond between the 2'-hydroxyl and pyrone-carbonyl groups. The low C=O frequency (1660cm^{-1}) ofdicoumarol was also revealed to have the strong intramolecular hyrodgen bonding between the two halves of the molecules.
C=C double bonds	Normally, there were three absorption bands at the region of 1600 to 1660 cm^{-1} in the standard spectra of dicoumarol. This pattern of absorption provides a quick method of differentiation from isomeric chromones in which the absorption is generally much simpler. There is an addition to the aromatic bands at ~1500 and ~1600 cm^{-1}, a strong sharp band appears at 1645 cm^{-1} which was attributed to the C=C stretching of the furan ring.

Table 5.4 : Continued

C-O single bond	The two bands found at the regions 1088 to 1109 and 1253 to 1274 cm^{-1} in the dicoumarol spectra were considered to be characteristics of stretching of the furan group. A weak band in the range of 1031-1188 cm−1 was assigned to the stretching frequency of C-O of lactone ring
Other absorptions	The bands at the 710 to 767 and 826 to 869 cm^{-1} region were due tothe in-plane and out-of-plane deformations respectively of the furanC-H bonds. The OH group was involved in H-bond formation and the band is weakly observed. There was also a strong vibration around 1108 cm^{-1} in both the compounds, which was assigned to the complementary band for the ketone C=O carbon.

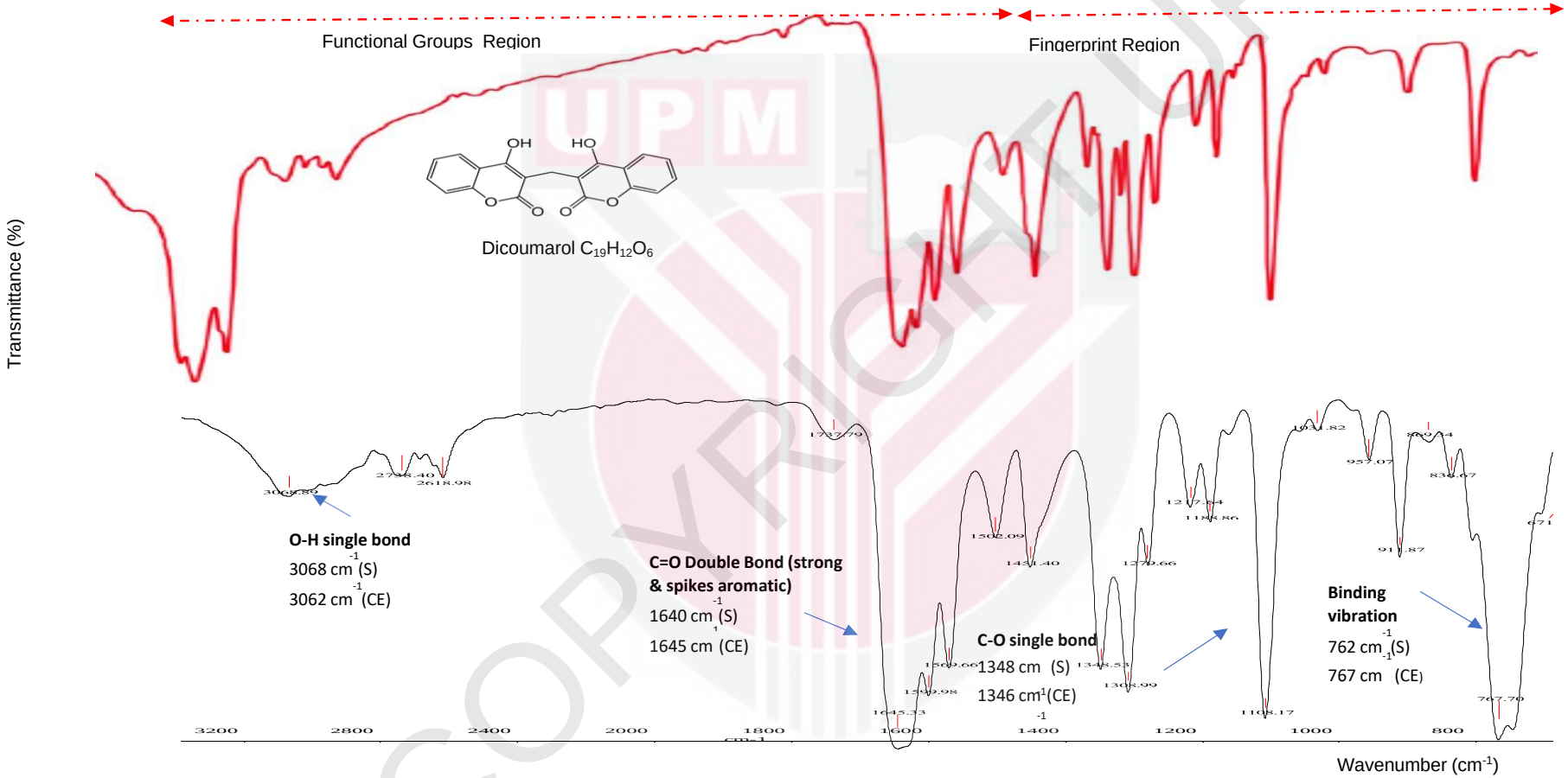


Figure 5.10 : Experimental FT-IR spectra of dicoumarol comparing standard (red lines) and sample fraction (black lines)

5.5.3 Characterisation of Dicoumarol Using Liquid Chromatography Mass Spectrometry/ Mass Spectrometry

The structure elucidation of dicoumarol was confirmed and quantified by Liquid Chromatography Mass Spectrometry/Mass Spectrometry (LCMS/MS). LCMS/MS enabledthe second MS with Electron Impact (EI) mode to generate a good fragmentation pattern to figure out the dicoumarol compound.

The X axis displayed the m/z, the abbreviation to denote the dimensionless quantity formed by dividing the mass number of an ion by its charge number in unit of Dalton (Da) while the Y axis is the intensity which measures the abundance of ions detected by the mass spectrometer. About four daughter ions spectra of dicoumarol weregenerated by collision-activated dissociation (CAD) at a collision offset of –10 V from the 70-eV electron impact (EI) with the highest peak of parent ion (M+ = 336 m/z).

The ESI (+) spectrum of dicoumarol showed the m/z 336. [M+H] + as base peak and the product ion spectra from the precursor yielded two intense fragment ions. At a collision energy of 25 eV, the m/z 79 ±2 $[C_5H_3O]^+$ was formed as 2-oxopropyl corresponding to theloss of the 4-hydroxy-2H-chromen-2 and at collision energy of 40 eV, the m/z 254 ±2 $[C_{14}H_9O_5]^-$ was formed (3,6-dihydroxy-8-methyl-9-oxoxanthen-1-olate) as shown in Figure 5.10.

The other peak of characteristic EI daughter ions were 199.0 and 139.2 m/z in dicoumarolsample fraction as shown in Table 5.5.

Table 5.5 : The highest peaks of theoretical and experimental precursor ion bytandem mass spectrum

Sample ID	Theoretical precursor ion m/z (±2 Da)	Experimental precursor ion m/z (±2 Da)	Theoretical product ion m/z (±2 Da)	Experimental product ion m/z (±2 Da)
Standard	336.063	338.919	NA 81.000 139.200	NA 81.092 139.00
Sample Fraction	336.063	336.965	199.000 257.200 335.000	196.950 254.800 336.965

The peak scan of molecular ion of 336.8 m/z concluded that the sample fraction was successfully identified as dicoumarol. This finding is equivalent with the one investigated by Yamini *et al*. (1995) in his study on dicoumarol production from mouldy sweet clover with 336.0 m/z experimental precursor ions. Isabele Fourel *et. al* (2010) also found that determination among 14 anticoagulant rodenticides, dicoumarol gave 335.0 m/z of precursor ion at 7 minutes of retention time.

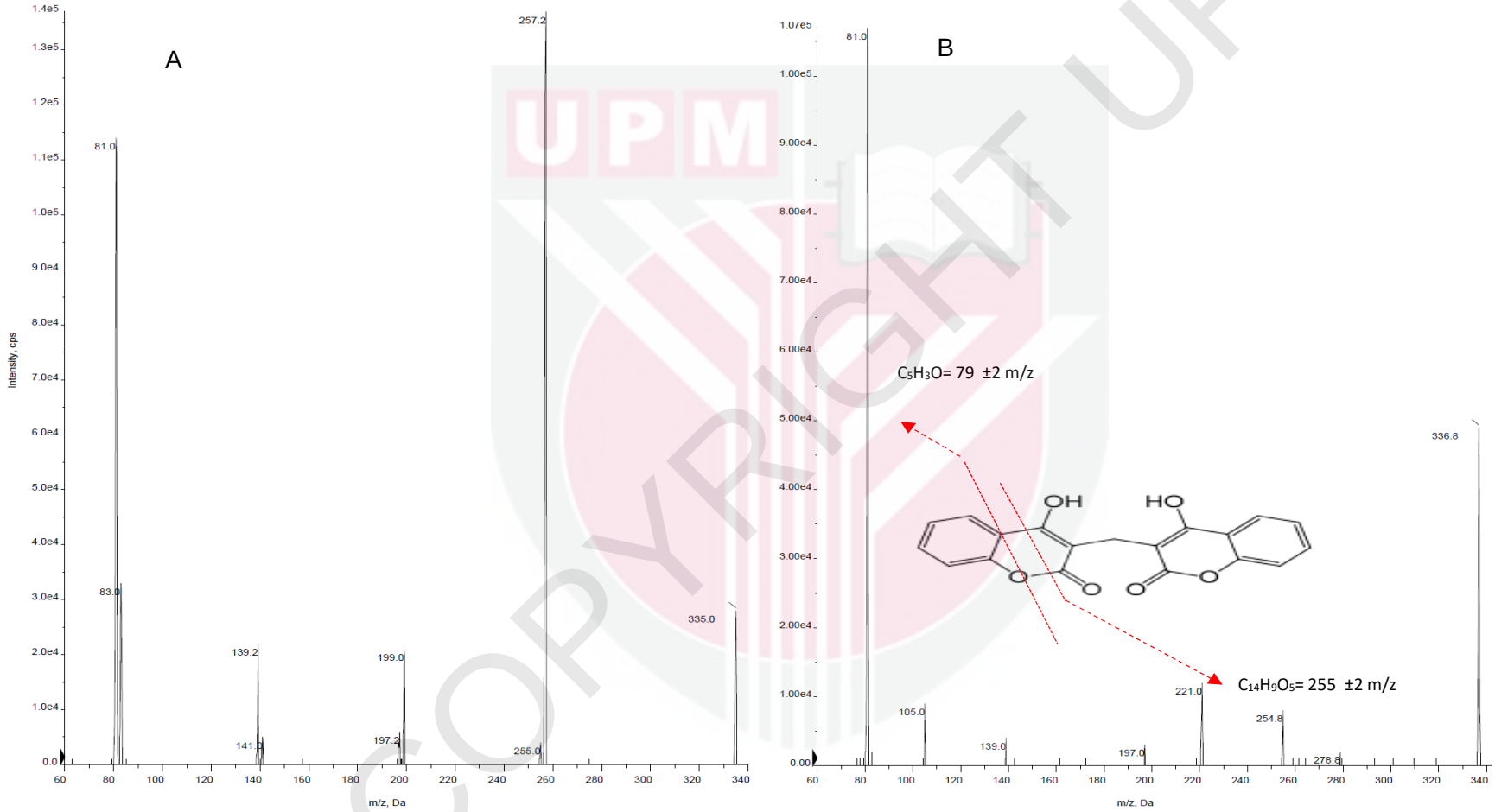


Figure 5.11 : LC MS/MS positive EI (70 eV) daughter ion spectrum resulting from argon collision activated dissociation at a collision offset of – 10 V (A) standard; (B) sample fraction

5.6 Screening of in Vitro Rodenticide Anticoagulant Activity

Dicoumarol was used as positive control and exhibited a significant anticoagulant activity,with PT higher than 60 s and negative control was 19.07 ± 0.32 s. Meanwhile, aPTT was 320 s in dicoumarol fraction with negative control as 120.26 ± 0.03 s. As can be observedin Figure 5.12, dicoumarol fraction was able to lengthen the coagulation time in aPTT test43.8% higher than crude extract, demonstrating its potential anticoagulant activity.

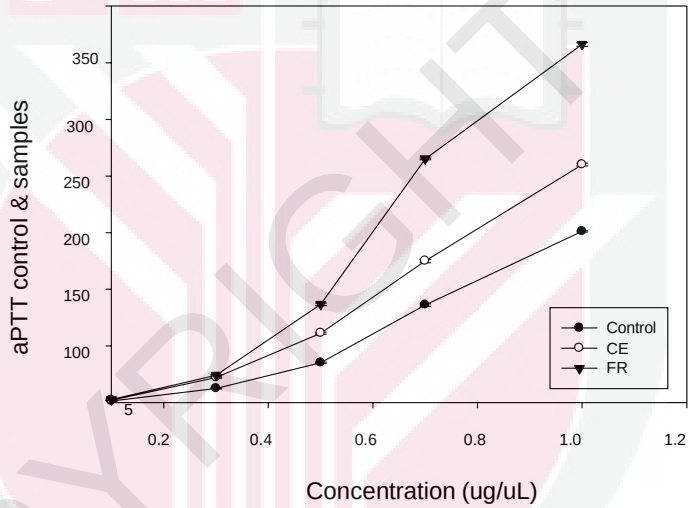


Figure 5.12 : Activated Partial Thromboplastin Time for control, crude extract (CE)and dicoumarol fraction (FR)

In PT test, no prolongation of the coagulation time was observed. The prolongation of aPTT indicates the inhibition of the intrinsic pathway of coagulation, whereas no prolongation of PT demonstrates poor inhibition of the extrinsic pathway (Li *et al.,* 2009). So, the present results suggest that dicoumarol fraction inhibits preferentially intrinsic pathways of coagulation. It is concluded that coumaric acid shows slight increase in anticoagulant activity while dicoumarol enhances the anticoagulation effect as been expected.

5.7 Chapter Summary

1. Bioconversion of dicoumarol by *A. fumigatus* NRRL 163 was greatly influenced bythe composition of medium with 10 g L^{-1} glucose and 2 g L^{-1} $NaNO_3$ concentrations. The optimal medium for dicoumarol production was defined media consisted of 5 g L^{-1} leaf extract, 5 g L^{-1} D-glucose, $NaNO_3$ (6g), KCl (0.52g), $MgSO_4.7H_2O$ (0.59g),KH_2PO_4 (1.52g) and trace elements of $FeSO_4.7H_2O$ (4mg), $ZnSO_4.7H_2O$ (0.4mg) were added into 1L deionized water with pH 4.8 (after sterilization).

2. The highest overall dicoumarol productivity (153.81 U mL h^{-1}), P_{max}/X_{max} (0.027 g L$cell^{-1}$) and overall dicoumarol productivity (4.15 U mL·h^{-1}) were obtained at 10 gL^{-1}glucoses. However, the highest maximum dicoumarol concentration (106.07 UmL^{-1}) was obtained at 2 g L^{-1} of $NaNO_3$ with P_{max}/X_{max} (0.38 g L $cell^{-1}$) and overall dicoumarol productivity (4.52 U mL h^{-1}). The dicoumarol production was inhibited at $NaNO_3$ concentration higher than 5 g L^{-1}.

3. The growth, substrate consumption and dicoumarol production by *A. fumigatus* NRRL 163 in batch fermentation was described through its kinetic studies. The maximum dicoumarol and cell concentrations obtained in batch fermentation usinga shake flask with cell efficiency of 37.13 g mL $cells^{-1}$ and overall dicoumarol productivity of 167.92 U mL h^{-1}. Dicoumarol was detected using HPLC at UV detection wavelength of 303 nm withretention time of 2.29 min which best match to the commercial standard.

4. Dicoumarol fraction demonstrated its potential anticoagulant activity with its abilityto lengthen the coagulation time in aPTT test 43.8% higher than crude extract. In PT test, no prolongation of the coagulation time was observed. It suggests that dicoumarol fraction inhibits preferentially intrinsic pathways of blood coagulation.

5. Bioconversion of dicoumarol from *o*-coumaric acid obtained from *G. sepium* leaves was successfully achieved and the converted compound was confirmed as dicoumarol through LCMS/MS with highest peak of parent ion (M+ = 336.8 m/z), confirming its molecular ions as 336.3 g/mol.

CHAPTER 6

CONCLUSION AND RECOMMENDATION FOR FURTHER WORKS

6.1 Conclusion

The following conclusions drawn from this study were fulfilled all the research objectives as;

1. The optimum drying temperature of *G. sepium* leaves was achieved at 50°C with 96% *o*-coumaric acid recovery. Higher yield of *o*-coumaric acid was obtained through UAE (28.95 mg L^{-1}) with 66.32% difference compared to SE (9.75 mg L^{-1}) respectively. Thin layer drying was successfully described the oven drying process of *G. sepium* leaves and the mathematical models of the drying kinetics was modelled using Midilli-Kucuk model.

2. The optimization of UAE using RSM was completely done with three parameters (temperature, time and ratio of solvent to solid). The optimum level of various parameters was obtained and it indicated that extraction temperature of 50°C, extraction time of 47 min and R_{ss} of 10:1 mL/g resulted to *o*-coumaric acid content of 36.14 ±0.015 mg L^{-1}. The predictive values matched well with experimental values with a good regression coefficient with high values of R^2 (0.9997) and adjusted R^2 (0.9941) indicating a high degree of correlation. The predicted correlation coefficient (0.9993) was also determined to be of high significance.

3. The bioconversion of dicoumarol by *A. fumigatus* NRRL 163 was greatly influenced bythe composition of medium, especially glucose and $NaNO_3$ concentrations. Defined media was found to be optimal medium for dicoumarol production consisted of 5 g L^{-1}leaf extract, 5 g L^{-1} D-glucose, $NaNO_3$ (6g), KCl (0.52g), $MgSO_4.7H_2O$ (0.59g), KH_2PO_4 (1.52g) and trace elements of $FeSO_4.7H_2O$ (4mg), $ZnSO_4.7H_2O$ (0.4mg) were addedinto 1L deionized water with pH

4.8 (after sterilization). The highest overall dicoumarol productivity (153.81 U mL h^{-1}), P_{max}/X_{max} (0.027 g L $cell^{-1}$) and overall dicoumarol productivity (4.15 U mL·h^{-1}) were obtained at 10 g L^{-1} glucose. The highest maximum dicoumarol concentration (106.07 U mL^{-1}) was obtained at 2 g L^{-1} of $NaNO_3$ with P_{max}/X_{max} (0.38 g L $cell^{-1}$) and overall dicoumarol productivity (4.52 U mL·h^{-1}). A Monod model based on First Order Reaction equations were fit to describe growth, substrate consumption and dicoumarol production by *A. fumigatus* NRRL 163 in batch fermentation. The maximum dicoumarol and cell concentrations obtained in batch fermentation using defined medium were 0.322 U L^{-1} and 9.33 g L^{-1} respectively.

4. *O*-coumaric acid was extracted from *G. sepium* leaves as the primary desired bioactive compound which was proven to be identical to the pure compound using HPLC with retention time of 2.781 min. The functional groups of compound were also confirmed through FTIR. Dicoumarol was successfully purified as secondary desired bioactive compound using HPLC fractionation with a single peak at retention time of 2.29 min and the ions fragmentation was achieved using LCMS/MS with the highest peak of molecular ion charge ratios of 336.8 m/z, indicating that the sample fraction was dicoumarol with molecular weight of 336.3 g/mol. The bioconversion process was successfully done.

6.2 Recommendation for Further Works

Although some improvements in dicoumarol production by *Aspergillus fumigatus Fresenius* have been achieved with the biological approach carried out in this study, refinement of the experiments and control strategies may be applied for further improvement as well as to get better understanding about the process. Some suggestions for future work are outlined as below:

1. Optimization of extraction process to achieve the optimum *o*-coumaric acid from *G. sepium* leaves shall be conducted using more efficient technique such as the use of other advanced statistical methods such as artificial neural network (ANN) and wavelet neural network (WNN). By using these techniques, three dimensional optimization exercises can be achieved.

2. The optimization of fermentation process could be further expanded using other parameters such as pH of media, agitation and could be scaled up through aerobic fermentation using 2L laboratory bioreactor for better cultivation of *A. fumigatus* NRRL 163. Optimization of bioconversion process will be carried out so that the optimum conditions of the fermentation process to produce higher cells density and maximum yield. Information on operating variables including dissolve oxygen, agitation and aeration mustbe studied to produce the best conditions for the maximum yield production. The result obtained from bioreactor system will be compared to shake flask system in term of yield production rate.

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APPENDICES

Table A.1 : Drying of 10g of G. *sepium* leaves at 50 °C

Time (min)	Weight (g)	Moisture Content	Moisture Ratio	Drying Rate
0	10	72.89	1	
10	8.837	61.26	0.840445	0.1163
20	7.953	52.42	0.719166	0.0884
30	7.07	43.59	0.598024	0.0883
40	6.333	36.22	0.496913	0.0737
50	5.474	27.63	0.379064	0.0859
60	4.745	20.34	0.279051	0.0729
70	4	12.89	0.176842	0.0745
80	3.329	6.18	0.084785	0.0671
90	2.953	2.42	0.033201	0.0376
100	2.811	1	0.013719	0.0142
110	2.743	0.32	0.00439	0.0068
120	2.729	0.18	0.002469	0.0014
130	2.721	0.1	0.001372	0.0008
140	2.719	0.08	0.001098	0.0002
150	2.715	0.04	0.000549	0.0004
160	2.713	0.02	0.000274	0.0002
170	2.712	0.01	0.000137	1E-04
180	2.711	0	0	0.0001
190	2.711	0	0	0
200	2.711	0	0	0

Table A.2 : Determination of Deff of G. *sepium* leaves dried at 50 °C

Time (min)	Moisture Ratio, MR	ln MR
0	1	0
10	0.837156	-0.17774
20	0.652448	-0.42702
30	0.476056	-0.74222
40	0.311871	-1.16516
50	0.236888	-1.44017
60	0.15332	-1.87523
70	0.083434	-2.4837
80	0.048021	-3.03611
90	0.018109	-4.01137
100	0.008987	-4.71195
110	0.007109	-4.94635
120	0.003219	-5.73859
130	0.002951	-5.8256
140	0.002951	-5.8256
150	0.001878	-6.27758
160	0.001476	-6.51874
170	0.001073	-6.8372
180	0.000805	-7.12488
190	0.000537	-7.53035
200	0.000402	-7.81803
210	0.000268	-8.22349

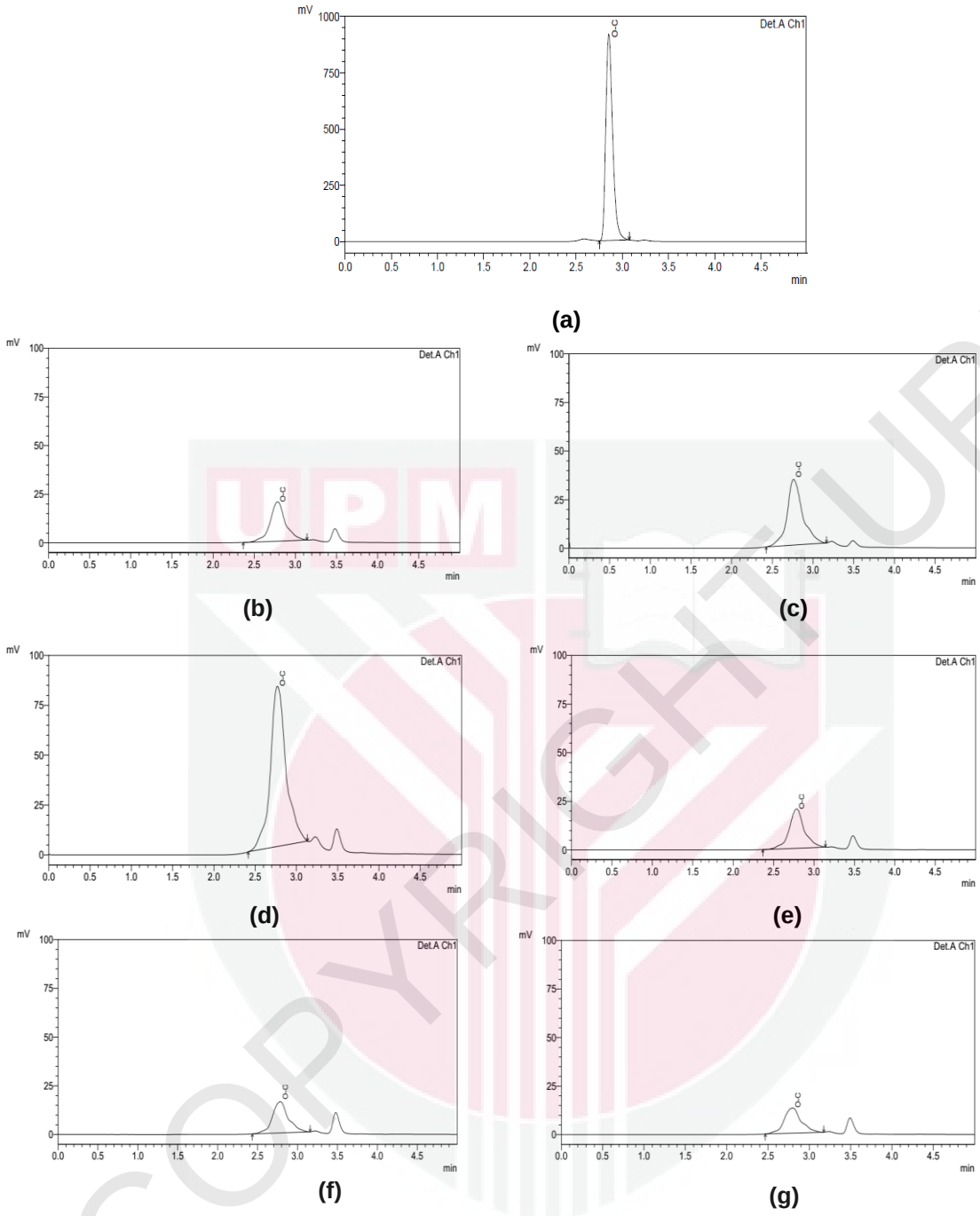


Figure A.1 : HPLC Chromatograms of standard (a), fresh leaf (b) and at differentdrying temperatures (c) 40˚C; (d) 50˚C; (e) 60˚C; (f) 70˚C; (g) 80˚C

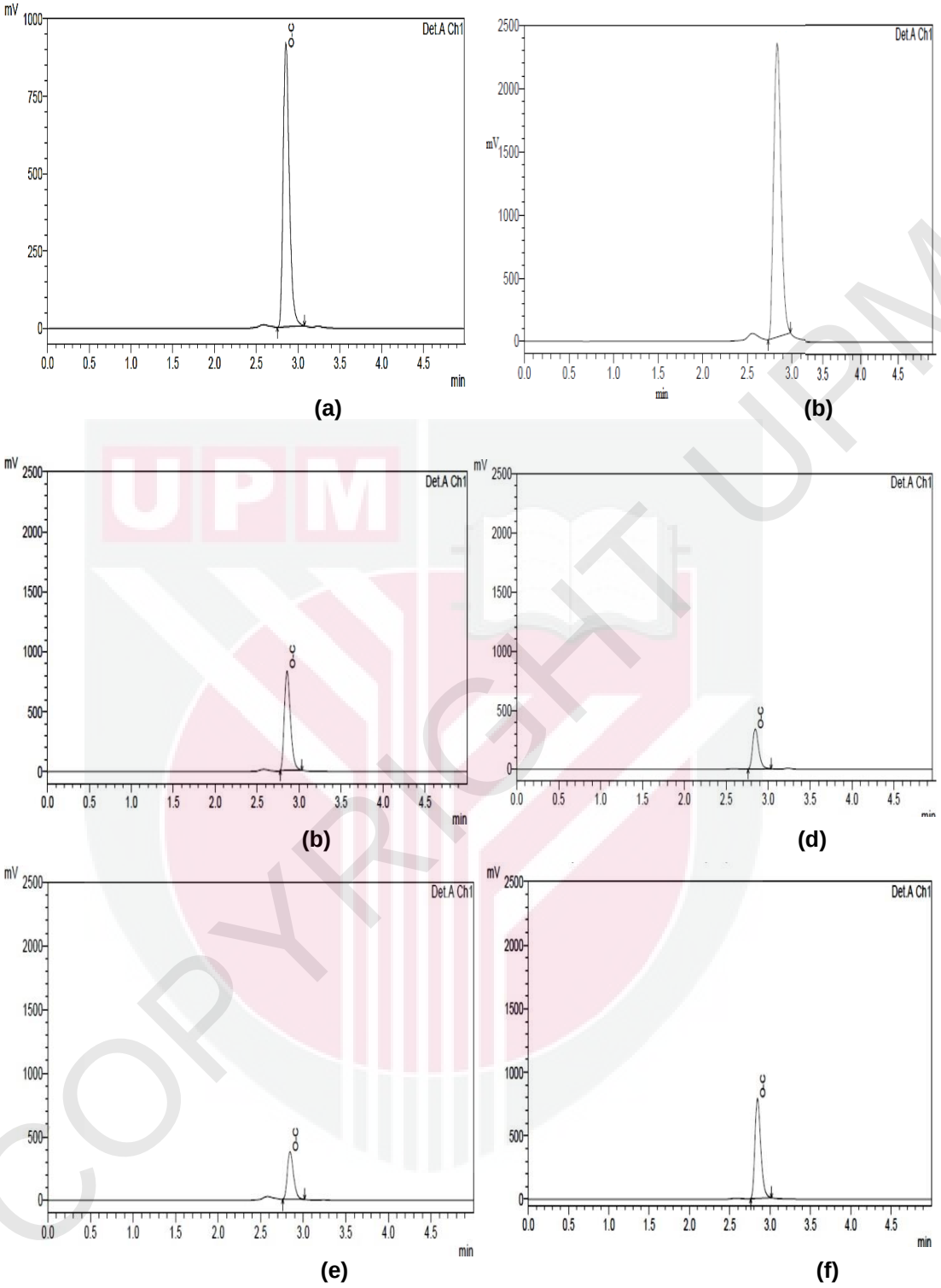


Figure A.2 : HPLC Chromatograms from UAE (a) Standard (10µL), (b) MetOH extract; Chloro extract; (d) EA extract; (e) H2O extract and (f) Hex extract

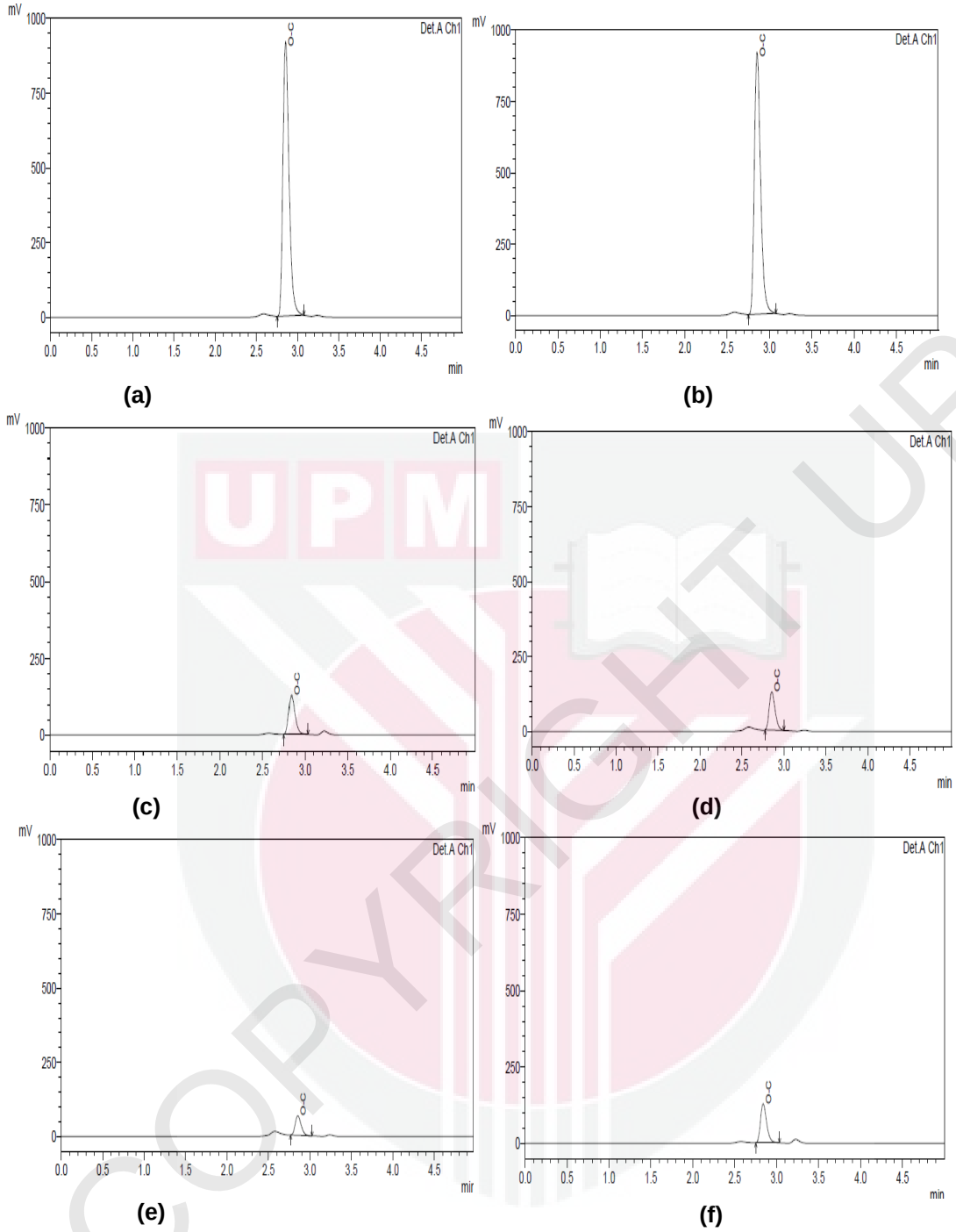


Figure A.3 : HPLC Chromatograms from SE (a) Standard (10µL), (b) MetOH extract; (c) Chloro extract; (d) EA extract; (e) H2O extract and (f) Hex extract

Table A.3 : Central composite design (CCD) plan in experimental and predictedvalues

Run	Independent Variables			Concentration of *o*-coumaric acid (mg/L)	
	A: Temperature (°C)	B: Extraction Time (min)	C: Solvent-Solid Ratio (mL/g)	Experimental	Predicted
1	50.01	40.00	5:1	26.84	26.92
2	50.01	50.00	5:1	34.03	34.12
3	60.00	50.00	5:1	9.930	9.930
4	60.00	40.00	5:1	10.47	10.37
5	47.00	45.00	10:1	36.14	35.92
6	55.00	45.00	10:1	15.20	15.37
7	55.00	45.00	10:1	15.20	15.37
8	55.00	45.00	10:1	15.20	15.37
9	55.00	45.00	10:1	15.50	15.37
10	55.00	45.00	10:1	15.50	15.37
11	55.00	45.00	10:1	15.50	15.37
12	55.00	53.00	10:1	10.09	9.970
13	63.00	45.00	10:1	9.430	9.530
14	50.00	40.00	15:1	16.37	16.46
15	50.01	50.00	15:1	18.73	18.91
16	60.00	40.00	15:1	9.270	9.270
17	55.00	45.00	18:1	10.04	9.920

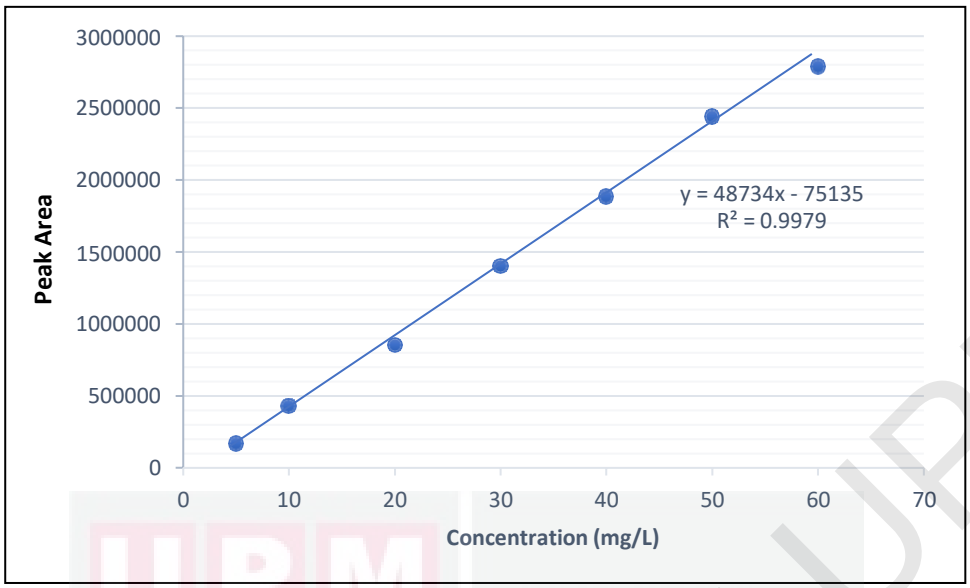


Figure A.4 : Calibration curve for *O*-Coumaric acid standard

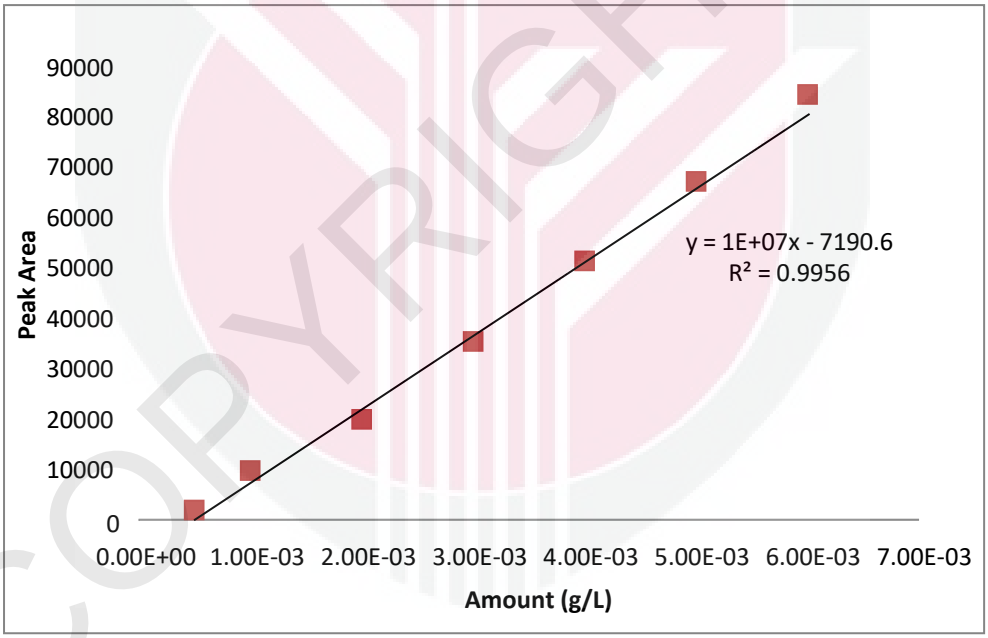
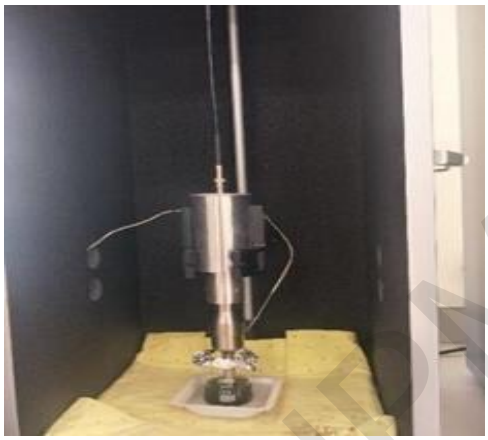


Figure A.5 : Calibration curve for dicoumarol standard

1. Leaves treatment beore oven drying and grinding



2. UAE extraction

3. Sample concentration using rotary evaporator

4. Collection of concentrated samples

5. Shake flask fermentation

6. Compound separation

Figure A.6 : Overall experimental process flow

LIST OF PUBLICATIONS

Siti Zalina Yaacob, Luqman Chuah Abdullah, Nor Hafizah Abdullah, Chuan Li Lee and Kit Ling Chin, Extraction Process of *O*-Coumaric Acid from *Gliricidia sepium* Leaves: Optimisation and Kinetics Studies, *Journal of Advanced Research in Micro and Nano Engineering*, accepted October 2024.

Luqman Chuah Abdullah, Siti Zalina Yaacob, Nor Hafizah Abdullah, Chuan Li Lee and Kit Ling Chin Optimization of Dicoumarol Production by *Aspergillus fumigatus Fresenius* using Methanolic Extract of *Gliricidia sepium* Leaves: Insights into Fermentation Kinetics Parameters Manuscript No: BAB-D-24-01477, *Biocatalysis and Agricultural Biotechnology*, submitted October 2024.

Siti Zalina Yaacob, Luqman Chuah Abdullah, Nor Hafizah Abdullah. 2017. Potential of *Trichoderma harzianum* as Cellulose Biodegrader in Biocomposting of Paddy Straw, *Journal of Advanced Research in Materials Sciences*, 39 (1); 8-13.