ANTIOXIDANT AND ANTI-ACETYLCHOLINESTERASE POTENTIAL FROM WASTE OF SELECTED SOLANACEOUS PLANTS: ISOLATION AND IDENTIFICATION OF THE ACTIVE COMPOUND

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Thesis submitted in fulfilment of the requirements
for the award of degree of
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Dedicated to
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ABSTRACT

Alzheimer's disease (AD) is one of the most common forms of dementia, affecting the elderly population globally. The occurrence of the ailment is on the rise; one person is being effected every 71 seconds. This rate is higher than many other fatal diseases, e.g., cancer, stroke, and heart failures. Therefore, searching for compounds with minimal side effects along with significant activity against acetylcholinesterase (AChE), are needed to be discovered to fight AD. In order to achieve this goal the study was undertaken to screen 32 medicinal plants based on their total phenolic contents (TPC), total flavonoid contents (TFC), antioxidant activity (AOA), and anti-acetylcholinesterase activity (AChE) to select the best source of the active compounds that can be used for the treatment of Alzheimer's disease. For the purpose, crude extracts from all 32 plants were subjected to determine TPC, TFC, AOA, and anti-AChE activity, and 3 plants out of 32 were finally selected for further study. The selected plants include *Solanum tuberosum* (Potato), *Solanum melongena* (Brinjals), and *Capsicum annuum* (Chilli). All three species belong to genus *solanum* and family solanaceae. TPC was determined following the Folin-Ciocalteu colorimetric method and TFC was determined using aluminium chloride colorimetric assay. AOA was determined by DPPH-scavenging and β-carotene assay methods, while anti-AChE activity was assayed by Ellman's method. A pure compound α-solanine was isolated by TLC, followed by HPLC and prep-HPLC. The fractions and isolated compound both showed significant bioactivities. The crude extract of *S. tuberosum* showed the highest anti-AChE activity (IC_{50} = 689.9 µg/mL), followed by, *S. melongena* (IC_{50} = 731.99 µg/mL), and *C. annuum* (IC_{50} = 851 µg/mL). Purified α-solanine demonstrated the second highest anti-AchE activity (IC_{50} = 725.70 µg/mL). A similar trend was seen for their antioxidant activity. In this case, TPC and TFC showed significant correlation with AOA and anti-AchE activity; attributing the bioactivities to be due to phenolic and flavonoids compounds present in plant fractions. The correlation between AChE, DPPH, TPC and TFC, were all found to be statistically significant ($P<0.05$). Furthermore, the finding shows that a good antioxidant compound can be a potent inhibitor of AChE. The data revealed that *S. tuberosum* fraction had higher activity compared to α- solanine that might be due to the synergistic effect of alkaloidal compounds and the polyphenols present in the crude extract. The structure of the isolated compound was elucidated by different chromatographic and spectroscopic techniques including HPLC, FTIR, NMR and LC-MS. The research results indicated that Solanum species could be the candidates of choice in further search for new AChE inhibitors.

ABSTRAK

Penyakit alzheimer's (AD) merupakan sejenis penyakit penurunan fungsi saraf otak yang menyerang populasi masyarakat warga emas dunia. Potensi mendapat penyakit AD semakin meningkat di mana seorang menghidapinya bagi setiap 71 saat. Ianya sangat tinggi jika dibandingkan dengan penyakit kanser, stroke and jantung. Kajian terhadap kompoun baru tanpa kesan sampingan dan mempunyai kekuatan tinggi untuk melawan acetylcholinesterase (AChE), dalam melawan AD adalah cabaran hari ini. Oleh itu objektif utama kajian ini adalah untuk mengenalpasti aktiviti antioksidan (AOA),perencat aktiviti acetylcholinesterase (AChE), jumLah kandungan phenol (TPC) dan jumLah kandungan flavonol (TFC) dan hubungan aktiviti stuktur (SAR) terhadap 2 genus solanum dan 1 genus capsicum untuk mengawal aktiviti AChE pada clift sipnatik. Untuk tujuan ini, ekstak mentah dan pecahannya digunakan sebagai bahan asas pemencilan kompoun aktif pada bahagian yang tidak boleh di makan yang terdapat pada *Solanum tuberosum* (ubi kentang), *Solanum melongena* (terung) dan *Capsicum annuum* (lada). Ketiga-tiganya daripada family solanaceae. Kaedah DPPH dan β-carotene digunakan untuk menilai AOA, manakala kaedah Ellman's untuk mengenalpasti aktivity AchE. TPC diuji melalui kaeadah Folin-Ciocalteu manakala kaedah kalorimetrik aluminium klorida untuk TFC. Kompoun alkaloid α-solanine telah dipencilkan menggunakan pelbagai teknik termasuk TLC, HPLC, HPLC preparative. Melalui hasil ujikaji, ekstak dan kompound yang dipencilkan menunjukkan respon positif terhadap ujian yang dijalankan. Ekstrak s*olanum tuberosum* menunjukkan aktiviti terbaik menentang AChE (IC50 = 689.9 μg/mL) diikuti oleh standard α-solanine (IC50 = 725.70 μg/mL), s*olanum melongena* (IC50 = 731.909 μg/mL), and *capsicum annuum* (IC50 = 851 μg/mL). Trend yang sama didapati pada ujian antioksidan. Untuk penambahan, struktur kompoun yang dipencilkan telah dirungkaikan melalui instumentasi HPLC, LC-MS, FTIR dan NMR. Untuk kompoun ini, AOA menunjukkan hubungan yang jelas dengan AChE, begitu juga TPC dan TFC di mana ia membuktikan aktiviti tersebut dipengaruhi oleh phenol dan flavonol yang terdapat pada tumbuhan tersebut. Sesuatu yang menarik menunjukkan ekstrak *S.tuberosum* menunjukkan aktiviti yang lebih tinggi berbanding kompoun standard. Ianya disebabkan kesan sinergi yang terdapat pada tumbuhan. α-chaconine dan α-solanine yang dikesani secara bersama dalam satu-satu tumbuhan didapati saling menyokong dan menunjukkan reaksi yang bagus. Kesemua hubungan adalah jelas dan menunjukkan keputusan antioksidant yang baik dan penghalang AChE yang bagus serta mempunyai keputusan TPC dan TFC yang memberangsangkan. Dicadangkan pada masa depan, kajian terhadap perencat AChE baru perlu diperluaskan terhadap mana-mana alkaloidal daripada famili solanaceae.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ATC	Acetylthiocholine
Abs	Absorbance
CNS	Central nervous system
FTIR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-mass spectrometry
HPLC	High pressure liquid chromatography
LC-MS	Liquid Chromatography mass spectrometry
LMIC	Low and middle income countries
NMR	Nuclear magnetic resonance
OD	Optical density
PNS	Peripheral nervous system
ROS	Reactive oxygen species
TLC	Thin layer chromatography
TMP	Transmembrane pressure

LIST OF SYMBOLS

$^{\circ}C$	Degree celcius
cm^2	Square centimeter
g	Gram
H	Height
h	Hour
min	Minute
mg	milligram
mL	milliliter
m/z	Mass to charge ratio
nm	Nanometer
t	Triplet
s	Singlet
d	Doublet

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

According to the World Health Organization (WHO), traditional medicine is the sum of total knowledge of skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. It has been estimated that in some Asian and African countries, 80% of the population depend on traditional medicine for primary health care while in many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine. Herbal treatments are the most popular forms of traditional medicine (WHO, 2008).

The traditional medicinal plants and functional foods are defined to have ingredients that have additional benefits over and above the normal one (Exarchou et al., 2006). The antioxidant activity of these plants is a part of particular interest, both because of their beneficial physiological activity in human cells as well as potential they have to replace the synthetic antioxidants used in medicine and food (Repetto and Llesuy, 2002).

Alzheimer's disease (AD) is regarded as one of the most common forms of dementia. It has affected an estimated 35.6 million people worldwide (Reitz et al., 2011). Geographical breakdown of the affected population is as follows: 7.82 million in the USA, 9.95 million in Europe, 1.86 million in Africa, and around 15.94 million people in Asia (WHO, 2012). Epidemiological studies indicate that these numbers are expected to grow at an alarming rate. It is estimated that the numbers will nearly double

up every 20 years, to 65.7 million in 2030 and 115.4 million in 2050 (Sousa et al., 2009). The majority of these people will be living in low and middle income countries (LMIC). Population ageing is having a profound impact on the emergence of the global dementia epidemic, influencing awareness and driving demand for services. It is reported that the numbers and proportions of older people will increase rapidly, especially in China, India and Latin America. By 2050, people aged 60 and over, will account for 22% of the world's population, of which 80% are living in Africa, Asia or Latin America (United Nations, 2003).

Currently, there is no proper cure for AD (Wolf-Klein et al., 2007). However, the inhibition of acetylcholinesterase is one of the most effective therapy with some consistent positive results (Holden and Kelly, 2002). There are four different drugs in the modern medicinal system, which are potent inhibitor of the enzyme including neostigmin, physiostigmin, Galantamine and memantine (Kumar et al., 2012). Further more, pathophysiology of AD is quite complex, and it involves several different biochemical pathways (Murlai, 2002). Reports suggest that oxidative stress may contribute to the pathogenesis of this disease (Foy et al., 1999). Therefor, the role of free radical scavengers or antioxidants should not be ignored in the treatment and prevention of AD.

The oxidation that occurs in the human body have negative consequences. Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide, formed in the body during metabolism are highly reactive and can cause damage to cellular structures, lipids, DNA and proteins (Silva et al., 2010). These oxidative processes, increase the risk of more hundred thirty different disease processes, of which dementia is one that includes Alzheimer's and Parkinson's disease (Aazza et al., 2011).

There is global interest in finding new and safe antioxidants from natural sources, to prevent oxidative deterioration of foods and to minimise oxidative damage of living cells. Antioxidants may act as chemical trape that absorb energy and electrons, quenching free radicals from carotenoids, anthocyanidins; catalytic systems that neutralize or divert reactive oxygen species. Antioxidant enzymes superoxide dismutase

(SOD), catalase, and glutathione peroxidasebinding to metal ions prevents the generation of free radicals (ferritin, ceruloplasmin, catechins); and chain-breaking antioxidants which scavenge and destroy reactive oxygen species of ascorbic acid, tocopherols, uric acid, glutathione, flavonoids (Karadag et al., 2009).

Therefore, based on their mode of action, the antioxidants can be classified as primary or secondary antioxidants. Primary antioxidants are able to donate a hydrogen atom rapidly to a lipid radical, forming a new, more stable radical, while secondary antioxidants react with the initiating radicals or inhibit the initiating enzymes, or reduce the oxygen level withoutgenerating reactive radical species. So the secondary antioxidants can retard the rate of radical initiation reaction by elimination of initiators (Miguel, 2010a). Since antioxidants can act through several mechanisms, the detection of such activity must be evaluated using various assays. In vitro, antioxidant assays in foods and biological systems can be divided in two groups: those that evaluate lipid peroxidation and those that measure free radical scavenging ability (Miguel, 2010b).

Studies have shown the significant results of treating the AD with antioxidants, and it is considered as a promising approach to treat the dementia. But it is affecting mostly to the extent that oxidative damage by free radicals, may be responsible for the cognitive and functional decline observed in AD. Although not a consistently regular observation, a number of epidemiological studies have found a correlation between antioxidant intake and reduced incidence of dementia, AD, and cognitive decline in elderly populations (Ringman et al., 2005).

1.2 PROBLEM STATEMENT

Nature has blessed Malaysia with abundant medicinal plants and currently, Malaysia is among the world's 12 mega biodiversity-rich countries, in terms of number of plant species. More than 20,000 plants species are found in the forest, of which, 2,000 species have been reported to have medicinal properties (Ang, 2004; Bakri, 2005). However, the exploration is very limited, as a result, many potential lead compounds would be awaiting for exploration.

AD is a neurodegenerative disease and its threat is getting higher than other fatal diseases like cancer, stroke, heart disease (Filho et al., 2006). Still there is no proper cure to handle it. A huge amount of money is being spent to deal with it. The four approved drugs have many side effects (Wolf-Klein et al., 2007). So, the need of the day is to come up with a new potential inhibitor by the natural products that control the activity of acetylcholinesterase with minimal side effects.

The major issue in the discovery of novel enzyme inhibitors is the cost factors, which are very high. The purpose of this study is to use in-vitro bench-top bioassays which will allow for the practical applications of bioassay-guided fractionation of crude extracts from plants and synthetic compounds in order to discover new pharmaceuticals. New enzyme inhibitors may be used to treat different neurodegenerative diseases.

1.3 RESEARCH OBJECTIVES

The goal of this study is to evaluate the bioactivity of a number of plants from different families and analyze the active plants on the basis of antioxidant and anti-acetylcholinesterase activity. Then, the active compound from most active plants will beisolated following the bioassays procedure. The following objectives were set to achieve this goal:

i) To screen 32 selected plants for AOA and anti-AChE activity and to evaluate the TPC, TFC, AOA, and anti-AChE, activity of the most active fraction.

ii) To fractionate the glycoalkaloidal compound from the non-edible part of the highest active plant.

iii) To isolate the alkaloidal compound from active fraction and elucidate the structure of the isolated compound.

1.4 SCOPE OF RESEARCH

Screening of 32 plants can help to find the best sources of bioactive compounds having antioxidant and anti-AchE activity. Solvent fractionation can give glycoalkaloids easily separated. HPLC can analyze the glycoalkaloid further. Each separate peak can be collected from the preparative HPLC. The pure fractions can be tested for their antioxidative, and anti-acetylcholinesterase activity. Finally, the structure of the active compound can be determined by FTIR, NMR, and LC-MS study.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

In this chapter literature review has been provided. This literature review has been categorised on the following subcategories. This approach has been adopted in order to present the vast literature in a concise manner which serves the purpose of this thesis. These categories are phytochemicals, natural products occurance, glycolakaloids applications, method of analysis and description of the studied species.

2.2 ANTIOXIDANT ACTIVITY

Antioxidant compounds in food play an important role as a health protecting factor. In food science, antioxidant was defined as a substance in foods that when present at low concentrations compared to those of an oxidizable substrate significantly decreases or prevents the adverse effects of reactive species, such as reactive oxygen and nitrogen species (ROS/RNS), on normal physiological function in humans (Huang and Prior, 2005). Plant antioxidants constitute one of the most active food compounds (Kris-Etherton et al., 2002; Surh, 1999). The first comprises chemical substances which interrupt the propagation of the free radical chain by hydrogen donation to radicals or stabilization of relocated radical electrons (Karadag et al., 2009).

The second group is characterized by a synergistic mode of action. It includes oxygen scavengers and chelators which bind ions involved in free radical formation. Their activity consists of hydrogen delivery to phenoxyradicals that leads to the reconstitution of the primary function of antioxidants. This role is played by substances

binding to metal ions, e.g. citric acid, and by secondary antioxidants, such as amino acids, flavonoids, β-carotene, selenium and many others. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants (Karadag et al., 2009). According to Akoh and Min (2008), the efficiency of phenolic free radical scavengers (FRS) depends on additional factors such as volatility, pH sensitivity, and polarity.

The concentration of antioxidants in raw materials depends, to a considerable extent, on the variety of crop plants and conditions of their cultivation. Kralova and Masarovicova (2006) also reported chemical changes and quantitative losses of selected groups of antioxidants and their biological activities in the entire processing chain starting with the raw material, through technological treatment, storage and culinary preparation until their absorption in the alimentary tract, all play an important role in the assessment of the antioxidant activity.

2.2.1 Phenolics

Phenolic acids known as multipurpose bioactive agents frequently occur in herbal plant (He, 2000). Phenolic compounds present in plant material have received considerable attention because of their potential antioxidant activity which play an important role in human nutrition as preventive agents against several diseases and protecting the body tissues from oxidative stress. Moreover the phenolic acids were considered in recent years as potentially protective compound against cancer and heart disease in part because of their essential antioxidant properties (Cartea et al., 2011).

All phenolic compounds have antioxidant properties, from simple phenols and phenolic acids possessing one phenolic ring to the complex polyphenols represented by the hydrolysable and condensed tannins (Harborne, 1998). Of notable antioxidant activity is α-tocopherol (vitamin E) a powerful chain–breaking antioxidant that inhibits lipid peroxidation (Esposito et al., 2002).

2.2.2 Flavonoids

Flavonoids are polyphenolic compounds that are widespread in nature and its implication for antioxidant activity is well known. Flavonoids as one of the most diverse group of natural compounds are probably the most important natural phenolics. The potential of dietary flavonoids has recently created an interest among scientist for treating many diseases (Kusirisin et al., 2009). These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties.

Flavonoids are characterized by having two benzene rings, usually phenolic, separated by a 3-carbon cyclic unit. Depending on the structure of this cyclic unit flavonoids are sub-classified into about ten sub-classes (Harborne, 1998). Important antioxidant sub-classes include flavones, flavanones, flavonols and isoflavones. Flavones such as quercetin are found abundantly in onions, apples and broccoli; flavanones in citrus fruit peel; catechin flavonoids in green and black tea; and anthocyanin reddish pigments in strawberries, roselle (karkade) and grapes (Esposito et al., 2002).

2.3 ANTIOXIDANTS AND ACETYLCHOLINESTERASE

Oxidative stress has been considered a mechanism involved in the pathogenesis of AD, and it has also played a major role in the aging process (Grassi et al., 2009). Oxidative damage by free radicals has been well investigated within the context ofoxidant/antioxidant balance (Madamanchi et al., 2005). Low levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important for maintenance of neuronal function, though elevated levels can lead to neuronal cell death. Antioxidants may scavenge ROS and may consequently attenuate inflammation pathways. Tertiary butyl hydroperoxide (tBHP) is a well established cytotoxin and oxidative stress inducer, and the rat pheochromocytoma cell (PC12) model is well suited for our research purposes. AD is known to be associated with amyloid-βplagues eliciting neuronal oxidative stress. Free radical damage has been identified as an underlying mechanism for specific neurodegenerative diseases. The brain consumes large amounts of oxygen and therefore produces a comparatively large amount of free radical by-

products. The increasing interest in the measurement of antioxidant activity of different plant samples is derived from the overwhelming evidence of the importance of ROS (Fernandes et al., 2004).

Acetylcholinesterase (AChE) is a 76 kD protein belonging to the α/β-hydrolase Family (Silman and Sussman, 2008). The catalytic site of AChE is found in a gorge which contains key amino acids responsible for the selective recognition of acetylcholine over all other molecules. These amino acids play a role in recognition of the tert-butyl group, basic nitrogen and the ester of acetylcholine (Marta, 1999). Three amino residues are key to the hydrolysis of acetylcholine. These amino acids include Glu327, which orients acetylcholine into the proper orientation within the active site so that it can abstract a hydrogen from a neighboring His amino acid. This His440 can then abstract a hydrogen from Ser200 making it a good nucleophile to attack the ester carbonyl carbon acetylcholine. This leads to choline being released and further introduction of a water molecule releases a molecule of acetic acid (Silman and Sussman, 2008).

2.3.1 Importance of Antioxidants

An antioxidant is defined as a molecule which 'when as present in a low concentration compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Sies, 1993). The two areas of commercial utilization of antioxidants this research project is most concerned with are applications to stop or reduce oxidative spoilage (oxidative rancidity) of crude or manufactured food products and intake of antioxidants, as ingredients of whole foods (nutraceuticals) or as food additives or even as drugs in their own right, to improve health and combat disease arising from oxidative stress. However, it must be noted that other opportunities exist for the use of antioxidants to stop oxidative spoilage in cosmetics, drug preparations and other commercial products prone to spoilage.

2.3.2 Antioxidants and Food Spoilage

Auto-oxidation occurs with food ingredients such as proteins and carbohydrates, however, it is more profound with unsaturated lipids i.e. lipids containing unsaturated fatty acids. In all cases the mechanism involves free radicals (species containing an odd electron) and passes through the characteristic three stages of the free radical mechanim viz., initiation, propagation and termination. In the case of unsaturated lipids, molecular oxygen in the air initiates oxidation by abstracting a hydrogen atom from an allylic position on the unsaturated fatty acid residue, generating an allylic radical. The latter reacts with another oxygen molecule forming a peroxy radical, which abstracts an allylic hydrogen from another fatty acid residue to form a hydroperoxide and anther allylic radical that propagates the chain. (Thenmozhi and Mahadeva, 2012). The unstable hydroperoxides generated decompose forming carbonyls that impart disagreeable odour and taste to the food. A phenolic moiety , whether it be part of the chemical structure of a synthetic antioxidant like BHT or of a natural one such as a flavonoid, can donate a hydroxyl-hydrogen atom to a radical generated during auto-oxidaion, 'neutralizing' it. The resonance-stabilized phenolic radical formed does not participate in further chain reactions of fat oxidation.

All foods that contain lipids are prone to oxidation, however, especially affected are those that are dehydrated or exposed to high temperature before storage, e.g. dehydrated eggs, cheeses and fried foods (Addis, 1986). Food oxidation is also of health concern as the radicals and oxidation products of substances like cholesterol are linked to the onset of certain diseases (Addis, 1986).

Historically, man used spices to prevent food deterioration. Spices and culinary herbs are good sources of antioxidants. Camo et al. (2008) reviewed this subject and reported that the aromatic herb rosemary (Rosmarinus officinalis) is much used by the food industry because of its strong antioxidant activity. Another antioxidant rich source is oregano (Origanum vulgare). Both herbs belong to the same family, Labiatae. Rosemary extracts were recommended for use with various foods, including beef steaks

and minced pork, at concentrations that ranged between 200 and 1000 ppm (Camo et al, 2008).

Anwar et al. (2007) reported successful preservation of sunflower and soybean vegetable oils by blending with moringa seed oil, which acted as a source of antioxidants. However, the fatty acid composition of the final blend changed. Banerjee et al. (2012) used broccoli powder extracts as a source of antioxidants to preserve nuggets made from goat meat. They reported that the natural extract, at the level of 2% was as good as the synthetic antioxidant, at 100 ppm. Serdaroglu et al. (2005) incorporated legume flours with meat to act as extenders in making meatballs. Descalzo and Sancho (2008) recommended using animal husbandry to deliver antioxidants to beef intended to preserve by making Argentinian cattle graze pastures high in the antioxidant α-tocopherol.

2.3.3 Antioxidants and Health

Free radicals are normal products of metabolism in human tissues. The main radicals generated in the human body are the superoxide and hydroxyl radicals. Other chemical species such as hydrogen peroxide and peroxynitrite, while not radicals themselves, are very reactive and can generate radicals through various reactions (Esposito et al, 2002). All these radicals and pro-radical species are referred to as reactive oxygen species (ROS). As mentioned above the human body has enzymatic and non-enzymatic antioxidant systems that operate to remove ROS. The production of ROS is dependent on biological and environmental factors such as exposure to ultraviolet light, X-ray, tobacco smoking, pollution, pesticides as well as taking certain drug medicaments (Wang et al, 2011). Problems arise when the production of ROS exceeds the ability of antioxidant systems to remove them, a condition referred to oxidative stress, since ROS can oxidize vital cellular components such as membrane lipids, proteins and DNA leading to disease (Ali et al, 2008). For example, neurodegenerative disorders such as Alzheimer's and Parkinson's diseases partially result from oxidative

damage to the brain, a tissue that consumes substantial amounts of oxygen and thus particularly vulnerable to oxidative damage (Esposito et al, 2002).

2.3.4 Antioxidants as Nutraceuticals

In addition to endogenous antioxidants such as glutathione and antioxidant enzymes, the human body makes use of dietary antioxidants to scavenge radicals. Natural products as radical scavengers was reviewed by Esposito et al (2002). The most important natural antioxidants carotenoids*:* which are tetraterpenes formed by the so-called 'acetate mevalonate' biosynthetic pathway in plants (Harborne, 1998). Their chemical structures are characterized by the presence of a comparatively large number of conjugated double bonds. They are mostly yellow, orange or red pigments responsible for the colours of carrot root and tomato skin. Carotenoids were shown to scavenge singlet oxygen and a number of ROS in vitro. Carotenoids such as β-carotene are the dietary source of vitamin A which also has antioxidant activity (Esposito et al, 2002), as shown in Figure 2.1.

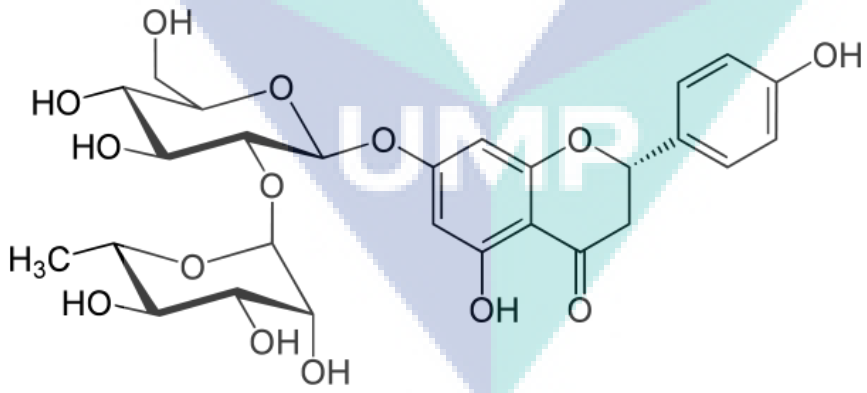
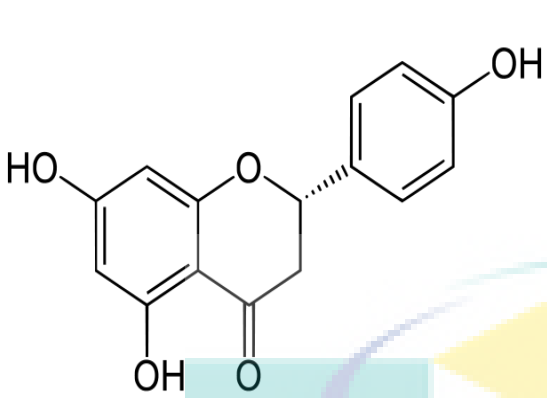
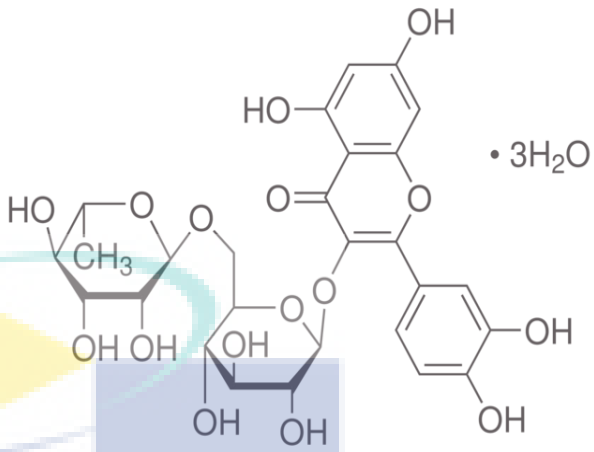


Figure 2.1: Naringin, a flavanone glycoside

Figure 2.1: Continued



Naringenin,a flavanone aglycone



Rutin hydrate, a flavonol

Unlike carotenoids, ascorbic acid (vitamin C) is water soluble and is a broad spectrum radical scavenger that removes peroxy and hyroyl radicals as well as superoxide, singlet oxygen and peroxynitrite (Esposito et al, 2002).

All phenolic compounds have antioxidant properties, from simple phenols and phenolic acids possessing one phenolic ring to the complex polyphenols represented by the hydrolysable and condensed tannins (Harborne, 1998). Of notable antioxidant activity is α-tocopherol (vitamin E) a powerful chain–breaking antioxidant that inhibits lipid peroxidation.

Perhaps the most important group of antioxidants is that of flavonoids (Mecocci and Polidori, 2012). Flavonoids are characterized by having two benzene rings, usually phenolic, separated by a 3-carbon cyclic unit. Depending on the structure of this cyclic unit flavonoids are sub-classified into about ten sub-classes (Harborne, 1998). It shows chemical structures of representatives of some of these sub-classes pertinent to this report. Important antioxidant sub-classes include flavones, flavanones, flavonols and isoflavones. Flavones such as quercetin are found abundantly in onions, apples and broccoli; flavanones in citrus fruit peel; catechin flavonoids in green and black tea; and anthocyanin reddish pigments in strawberries, roselle (karkade) and grapes.

Other potential sources of antioxidants have been examined. Peptide hydrolysates resulting from the hydrolysis of proteins obtained from eggs, fish, and several plants including soybean are gaining much research interest as potential antioxidants (Samaranayaka and Li-Chan, 2011).

On world-wide scale a large number of higher plants has been investigated as sources of natural antioxidants that lack the hazards associated with use of synthetics like BHA and BHT. It is logical that research efforts concentrated on edible plants. Spices represent one of the most researched group of plants and research uncovered rich antioxidant sources such as rosemary, sage, origanum, black pepper and turmeric (Suhaj, 2006). However, due to their strong flavour only limited amounts could be taken in the diet if they were to be useful nutraceuticals. Other important non-spice antioxidant sources uncovered include tea, walnut and betel nut (Suhaj, 2006). Fruits, vegetables and legume seeds (pulses) are now generally regarded as the best antioxidant nutraceuticals. Hafidh et al (2009) reviewed the results of scientific research carried out on antioxidant plant sources in Asia during 2000-2008, revealing that a total of 76 sources had been discovered. However, these authors stressed that more scientific work on this aspect is on demand.

A number of Malaysian higher plants have been investigated as antioxidant sources. For example, Said (2009) showed that several leafy vegetables (ulam) belonging to the taxonomic genera Cosmos, Ocimum, Vigna, Parkia, and others, possessed antioxidant and anti-tyrosinase activities. Ismail et al (2004) studied antioxidant activity and content of phenolics in a number of common vegetables including kale, spinach, cabbage and shallots, as affected by thermal treatment. The same research group (Ismail et al, 2009) also reported similar work on the non-leafy vegetables (fresh green pods) of the winged bean (*Psophocarpus tetragonolobus*), fresh bean (*Phaseolus vulgaris*), string bean (*Vigna sinensis*) and snow pea (*Pisum sativum*).

2.4 NATURAL PRODUCTS

Natural products have been used as a major source of cure for centuries. Natural products are secondary metabolites derived from pre-cursors of primary metabolism (Gonzalez-Molina et al., 2010). Secondary metabolites include alkaloids, terpenoids, steroids, phenols, and phenolics, do not play any role in the growth and development of living organisms; however, primary metabolites which include carbohydrates, proteins, and lipids are the building blocks of life. These, secondary metabolites have shown interesting pharmacological activities and are used in treating many human ailments. The mentioned compounds have a wide range of biological activities including anti-cancer, anti-leishmanial, anti-microbial and anti-viral (Schmelzer and Gurib-Fakim, 2008). The amazing bioactivities of natural products have attracted organic chemists to identify these bioactive constituents from natural sources and to synthesize them as well (Fernandes et al., 2007). More than 25% of the pharmaceutical drugs used in the world today are derived from plant natural products (Lubbe and Verpoorte, 2011).

2.4.1 Types of Natural Products

The sources of the natural products include plants, marine microorganism, microorganism and even insects. The compounds obtain from them can be classified into various classes on the basis of their structural features and biosynthetic origin. Natural products origin can be traced by the key intermediates of primary metabolism. Some of the intermediates include acetyle-CoA **(15)**, shikimic acid **(16)** and mevaolic acid **(17)**. Other groups of natural product contain alkaloids, nitrogen containing compounds mainly derived from amino acids, e.g. tryptophan **(18)** and L-ornithine **(19)**. The chemical structure of all are shown in Figure 2.2. Another group of natural products can originate from sugars and amino acids (Miklos and Jonathan, 2003).

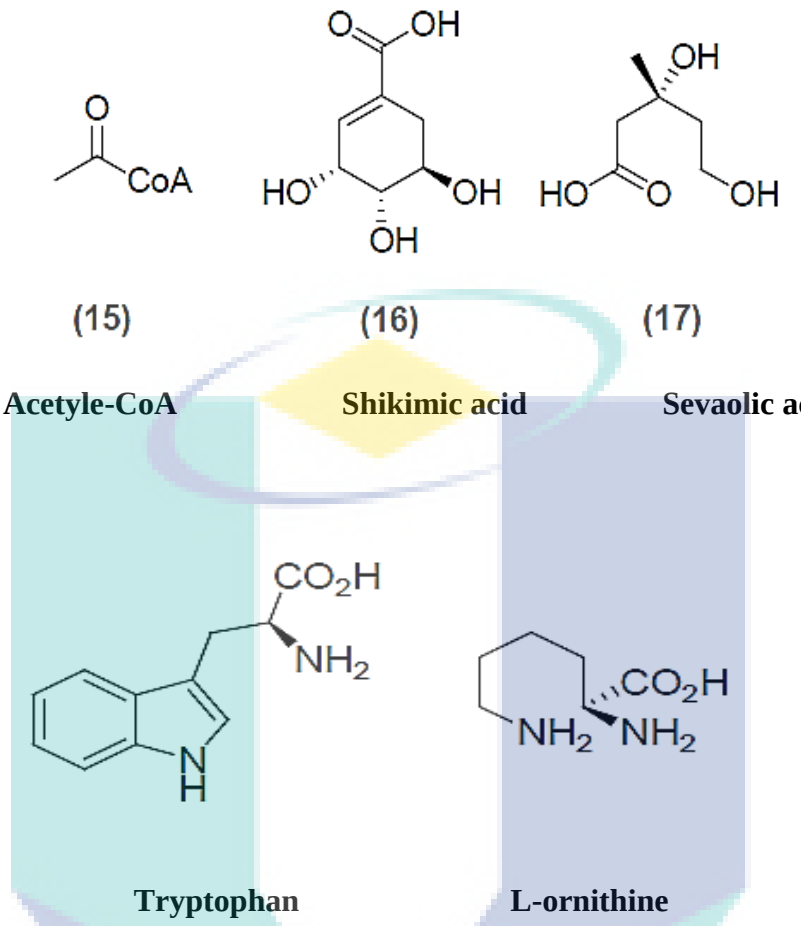
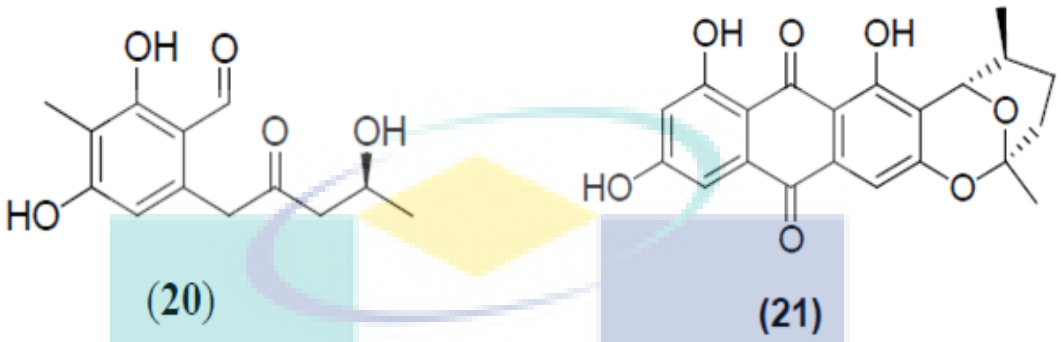


Figure 2.2: Some key metabolites of primary metabolism.

2.4.2 Polyketide Derived Natural Products

Polyketides derived from acetyl-CoA **(15)** come about from modifications in the fatty acid biosynthetic pathway. In normal fatty acid biosynthesis two molecules of acetyl-CoA undergo a Claisen reaction to form a ketoester. The pathway continues with a reduction to a hydroxyl group, followed by dehydration to a double bond and a final reduction to a reduced ester. This process is repeated many more times to form fatty acids. In polyketide synthesis, the process can be stopped at any of the steps and a further (Daniel et al., 2007).

Numerous isolated polyketides have shown to be pharmacologically active, such as 2,4-dihydroxy-6-((*R*)-4-hydroxy-2-oxopentyl)-3-methylbenzaldehyde **(20)** and averufin **(21)** (Bringmann et al., 2009). Figure 2.3 shows two active polyketides.



2,4-dihydroxy-6-((*R*)-4-hydroxy-2-oxopentyl) -3-methylbenzaldehyde **Averufin**

Figure 2.3: Pharmacologically active polyketide molecules.

2.4.3 Triterpenoidal and Steroidal Natural Products

Triterpenes are formed when two molecules of farnesyl pyrophosphate (FPP) **(22)** are joined tail to tail to give squalene **(23)**. Once squalene is epoxidized and folded into the proper orientation, it undergoes cyclization to yield a protosteryl cation **(24)**. This cation undergoes successive Wagner-Meerwein 1,2-hydride and methyl shifts to yield lanosterol **(25)** in animals and fungi and cycloartenol **(26)** (Atta-ur-Rahman et al., 2002), in plants, it can be seen below in Figure 2.4.

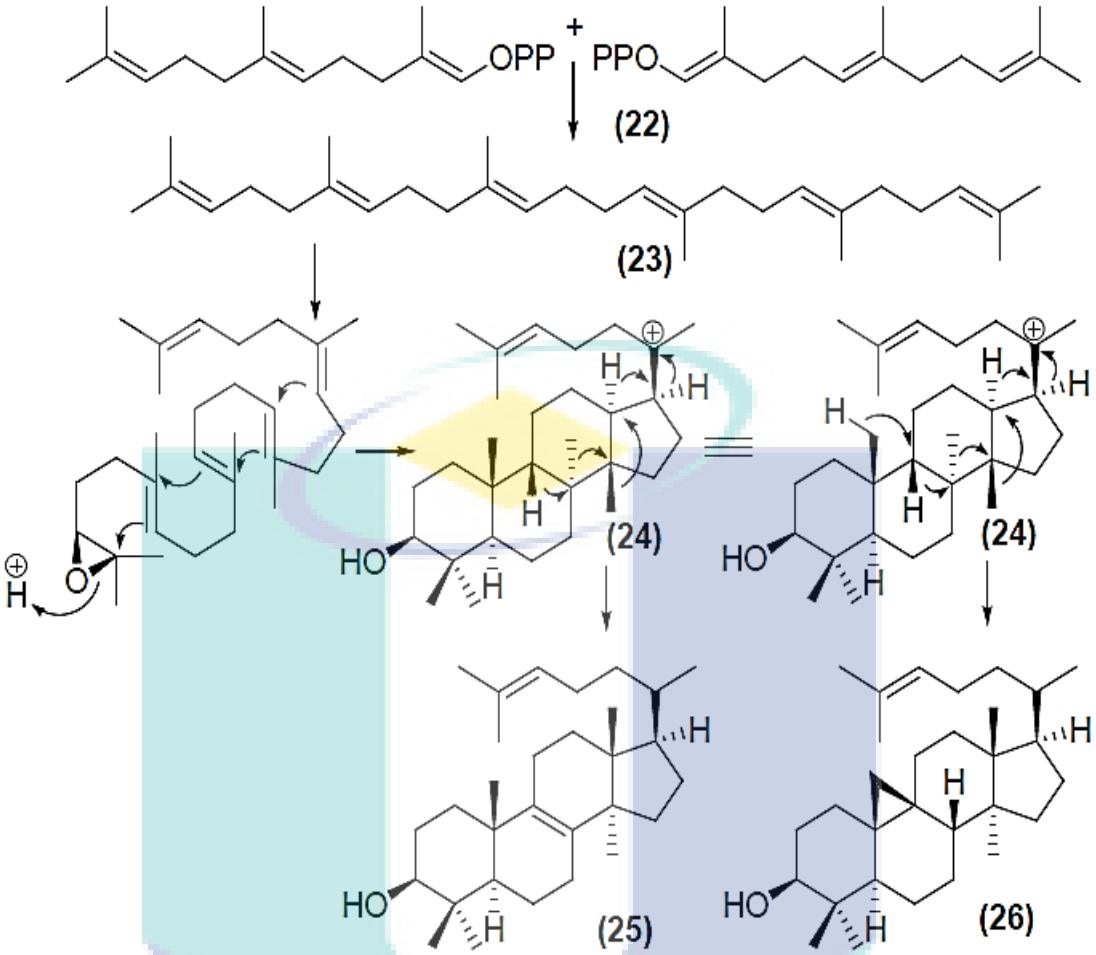


Figure 2.4: Triterpene skeleton biosynthesis.

2.4.4 Alkaloidal Natural Products

Alkaloids consist of nitrogen containing natural product class with a wide variety of features in the structure. This class can be further divided into subclasses. Some of these subclasses include Ornithin (**19**) derived natural products. In animals, L-Ornithine, is formed from the urea cycle, in plants it is synthesized from L-glutamic acid (**27**). Decarboxylation can proceed to putrescine (**28**), a natural product substituent *p*-coumaroylputrescine (**39**). Putrescine (**28**) can be further modified to *N*-methyl-Δ1-pyrrolinium cation (**29**), which can be used in the synthesis of tropine (**30**) a precursor to atropine (**2**) (Stobiecki et al., 2003), as seen in Figure 2.5.

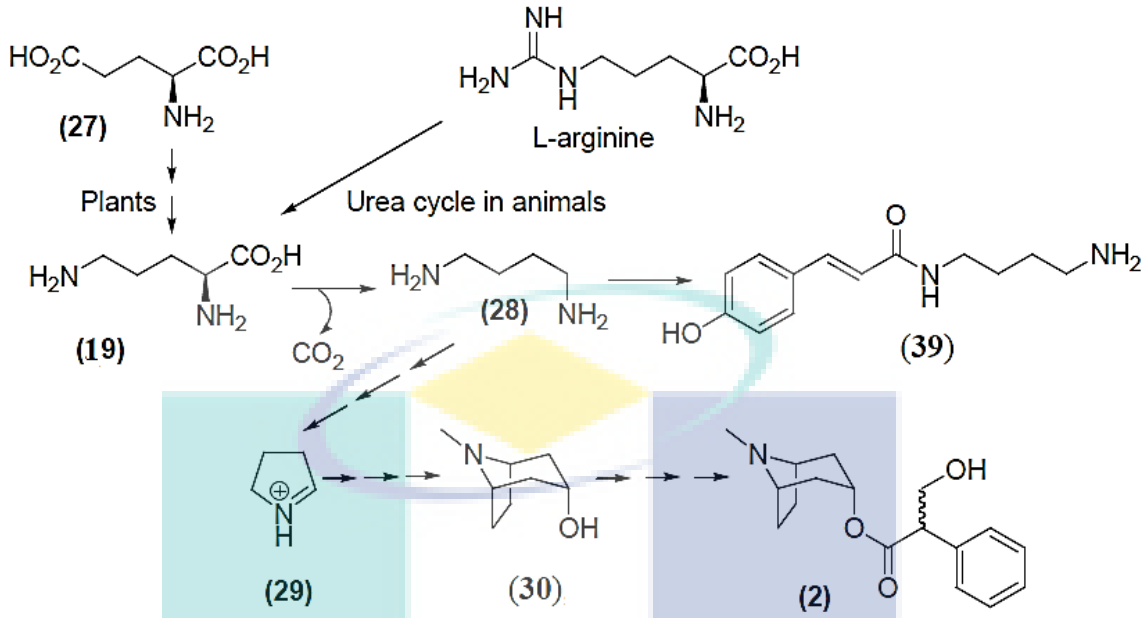


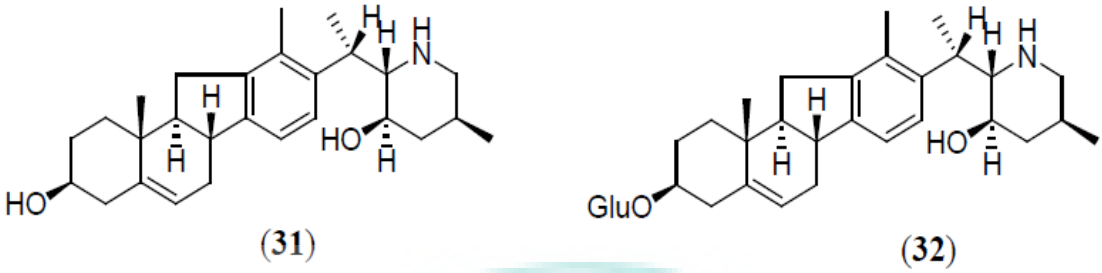
Figure 2.5: Biosynthesis of alkaloids.

Source: Dewick (2009)

2.4.5 Steroidal Alkaloids

Repeatedly found a class of alkaloids in plants, fungi and marine organisms is the "steroidal alkaloids". It possesses biological activities like anti-microbial and anti-cancer. This class has been found effective for the treatment of Alzheimer's disease (Kahn et al., 1997).

Steroidal alkaloids showing anti-cancer activities have been isolated from numerous sources, including common plants such as lilacs. Veratramine **(31)**, and rubijervervine **(32)** shown in Figure 2.6, isolated from the roots of a lilac plant (*Veratrum nigrum*) are examples that show weak cytotoxicity against brain tumours (SF-188 cell line) (Sibbesen et al., 1995).



(31) (32)

Veratramine **Rubijervervine**

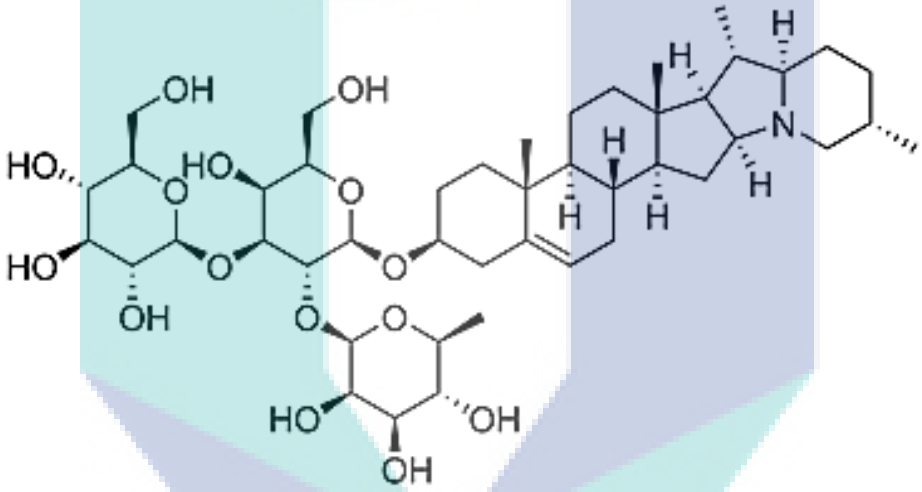
Figure 2.6: Anticancer steroidal alkaloids.

2.5 GLYCOALKALOIDS AND ITS ORIGIN

Glycoalkaloids are steroidal nitrogen–containing metabolites found in many *solanaceous* plants (Pia et al., 2009). Steroidal glycoalkaloids are found in almost all parts of the potato. The Highest concentration is found in the tissues which are undergoing metabolically (Kue, 1999). These include flowers, young leaves, unripe berries, sprouts, peels, and the area around the eyes. Small immature tubers are normally high in glycoalkaloids since they are still metabolically active (Papathanasiou et al., 1998). Glycoalkaloids are concentrated in a 1.5 to 3.0 mm layer immediately under the skin in normal tubers (Pęksa et al., 2006). The two major glycoalkaloids in potatoes are α-solanine and α-chaconine (Figure 2.7), which together comprise approximately 95 % of the total glycoalkaloids in the plant (Edwards and Cobb, 1999). The ratio of α-solanine to α-chaconine differs depending on the anatomical part of the potato plant or its variety, and ranges from 1:2 to 1:7 (Bejarano et al., 2000). The other glycoalkaloids found in cultivated potatoes are β- and γ-solanines and chaconines, α- and β-solamarines, demissidine, and 5-β- solanidan-3-a-ol, and in wild potatoes leptines, commersonine, demissine, and tomatine (Lachman et al., 2001).

Glycoalkaloids, a class of nitrogen-containing steroidal glycosides are naturally occurring secondary metabolites commonly found in the Solanaceae family which includes many significant agricultural plants, such as tomato, potato, eggplant, pepper, nightshade, thorn apple, and capsicum. For example, solasodine, has been found in

about 200 Solanum species (Dinan et al., 2001). Glycoalkaloids are generally found in all plant organs, with the highest concentrations occurring in flowers, sprouts, unripe berries, young leaves or shoots (metabolically active parts) (Friedman et al., 2005). Due to defensive character, development of new cultivars of tomato and potato with high foliar steroidal glycoalkaloid levels is underway. The types of steroidal glycoalkaloids produced by solanaceous plants vary from species to species. The differences can be shown by the presence or absence of a C-C double bond, a variety of functional groups (e.g., hydroxyl, acetyl) and sugar groups, as well as in the stereochemistry of these functional groups (Chen and Miller, 2001). The most common *Solanum* glycoalkaloids are given in Table 2.1.

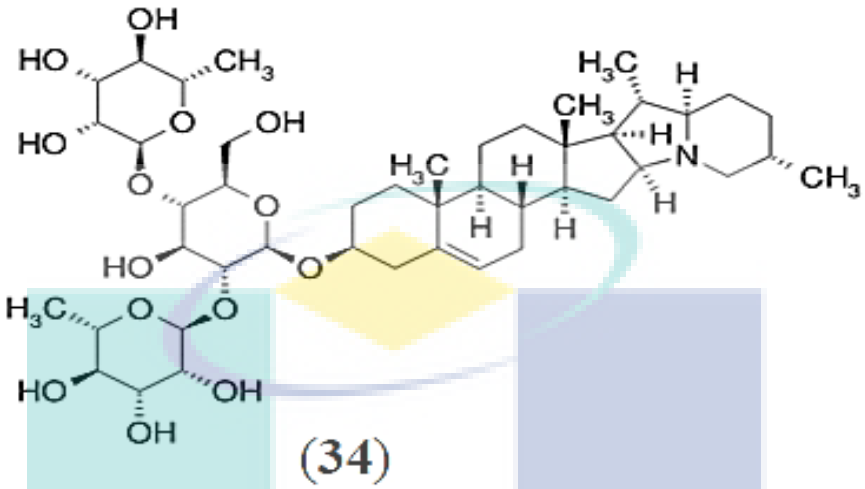


A-Solanine

Figure 2.7: Most common glycoalkaloids found in solanum species, especially in *solanum teberosum*.

Source : Peksa et al. (2006)

Figure 2.7 continued



(34)

Chaconine

Table 2.1: The most common glycoalkaloids found in solanum species (aAglycone, bR=aglycone; Gal= β-D-galactose; Rham: α-L-rhamnose; Glu: β –D glucpse;bXyl= β -D-xylose,cMinor SGAs may be artefacts or metabolites)

SGA	Sugar Moiety	Glycoside structure
Solanine Glycoside		
α- Solanine	Solatriose	A:R-Gal-Glu
β- Solanine [C]	Solabiose	B:R-Gal-Glu
γ- Solanine [C]	Galactose	C: R-Gal
α- Chaconine	Chacotriose	D:R:Glu-Rham
β_2- Chaconine [C]	Chacobiose	F:R-Glu-Rham
γ- Chaconine [C]	Glucose	G-Glu
Dehydrocommersoninse	Commertetratose	H:R-Gal-Glu-Glu
Demissideline glycoside		
Demissine	Lycotetrose	I:R:Gal-Glu- Glu
Commersonine	Commertetratose	R-Gal-Glu-Glu
Acetylleptinide glycosides		

Table 2.1 Continued

Leptine I	Chacotirose	R:Glu-Rham
Leptine II	Solatriose	R:Glu-Rham
Tomatidenol glycosides		
α-Solamarine	Solatriose	R-Gal-Glu
α -Solamarine	Chacotriose	R:Glu-Rham
Solasodine glycosides		
Solasonine	Solatriose	R-Gal-Glu
Solamargine	Chacotriose	R:Glu-Rham
Tomatidine glycosides		
α- Tomatine	Lycotetrase	A:R-G-Glu
Sisunine (neotomatine)	Commertetratose	R-Gal-Glu-Glu

Source: Laurila (2004)

2.5.1 Structure of Glycoalakloids Found in Solanum Species

The characteristics of steroidal alkaloid is the presence of intact or modified steroid skeleton with nitrogen. As nitrogen is inserted into a non-amino acid residue, these compounds belongs to a subgroup of pseudoalkaloids (or isoprenoid alkaloids) (Laurila, 2004). Structural variation in the family of plant steroidal glycoalkaloids is limited to two main groups, based on the skeletal type of the aglycone, examples of which are represented in Figure 2.8. One is the spirosolan type, similar to spirostan, but with nitrogen in place of the oxygen in ring F (forming a tetrahydrofuran and piperidine spiro-linked bicyclic system), as in solasodine **(35)**. Second is the solanidane **(36)** type, where N connects spirostan rings E and F rings. All types can contain double bonds and hydroxyls in various positions. At least 90 structurally different steroidal alkaloids have been found in over 350 *Solanum* species (Laurila, 2004). Nitrogen can be attached as a primary NH_2 group in position 3 or 20 (free or methylated), forming simple steroidal bases (e.g., conessine), ring-closed to skeletal or side-chain carbon (as a secondary NH) or annelated in two rings as a tertiary N **(36)**. This often influences the chemical character of the compound (Dinan et al., 2001).

Glycoalkaloids in the plants often found as glycosidic form. So, steroidal glycoalkaloids consist of three parts: a non-polar steroid unit and a basic portion with either a so called indolizidine or oxa-azaspirodecane structure which together form the aglycone part; a polar, water-soluble sugar moiety with three or four monosaccharides attached to the 3-OH group of the first ring of the aglycone. The common glycoalkaloid aglycones in eggplant and potato tubers are presented in Figure 2.8. (Solatriose group, Chacotriose group).

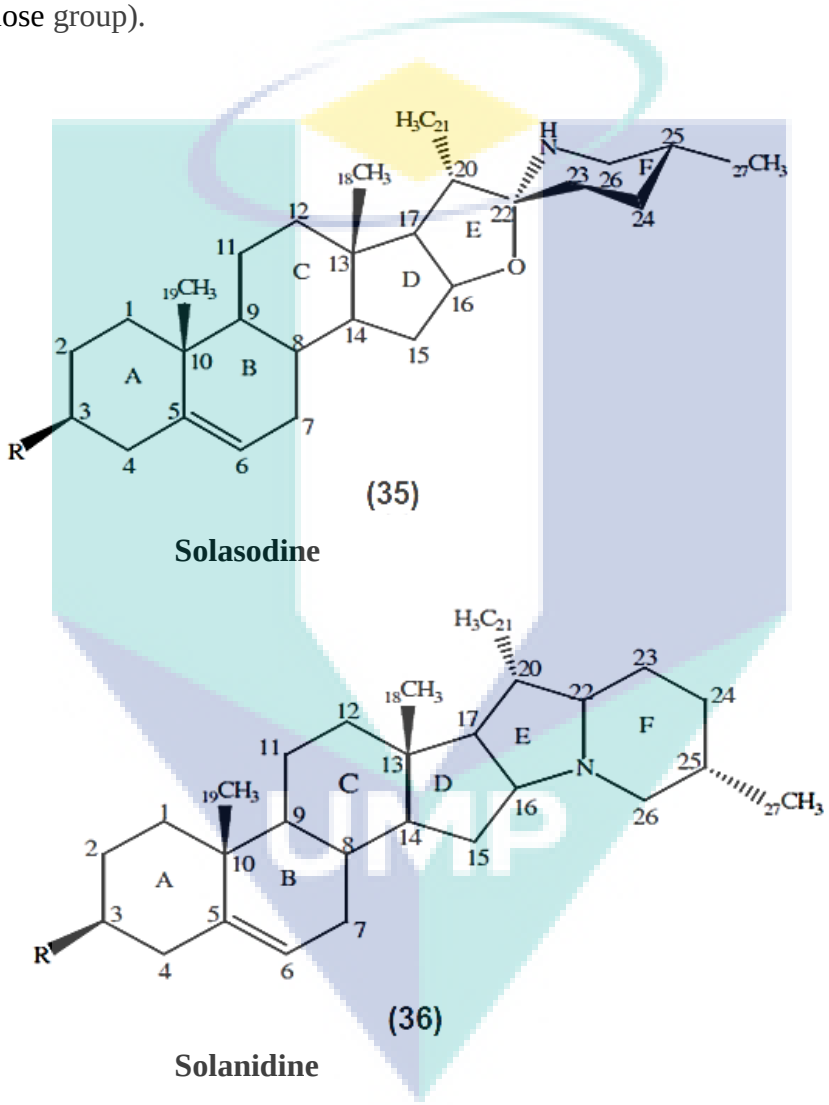


Figure 2.8: Glycoalkaloids found in solanum species.

Source: Chen and Miller (2001)

Solasonine and solamargine are the two major steroid alkaloid glycosides (SAGs) found in eggplants (*Solanum melongena*) and in at least 100 other Solanum

species (Ibarrola et al., 2011). Structurally, these two glycoalkaloids have the same steroidal part of the molecule (aglycone), solasodine, but differ in the nature of the carbohydrate side chain. The trisaccharide chain attached to the 3-hydroxy group of solasonine has a solatriose (branched a-L-rhamnopyranosyl-b-D-glucopyranosyl-bgalactopyranose) structure **(37)**. The corresponding trisaccharide of solamargine has the structure of chacotriose (branched bis-a-L-rhamnopyranosyl-b-glucopyranose) **(38)**, as shown in Figure 2.9. The most prevelant glycoalkaloids are A-solanine and a-chaconine, found in cultivated potato with solatriose and chacotriose sugar moiety respectively which are attached to aglycon solanidin. The glycoaalkaloids found in eggplant differ from potato glycoalkaloid only in the steroidal part structure of molecules. It has been reported that while solamargine and solasonine are in eggplant fruits, solanine and chaconine are found in the leaves of these plants (Shetty et al., 2011).

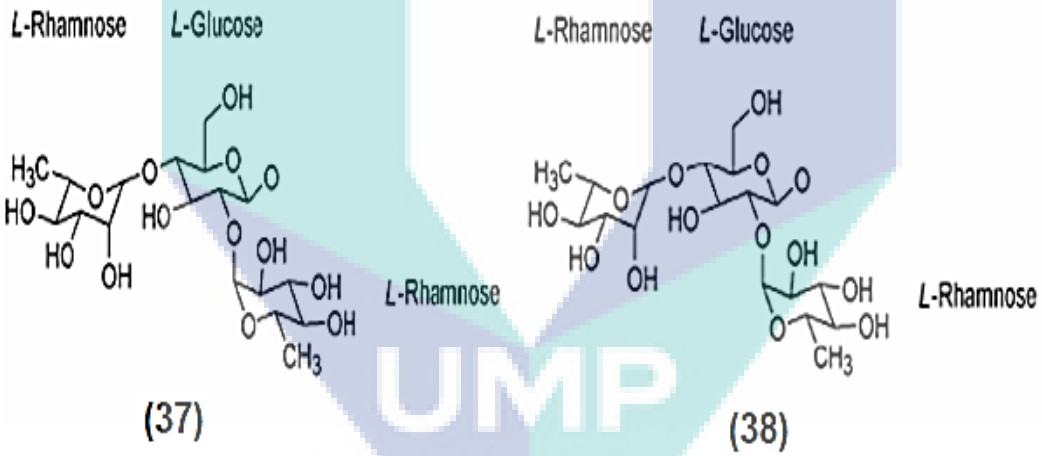


Figure 2.9: The trisaccharides of the glycoalkaloids.

Source: Alt et al. (2005)

2.5.2 Toxicity of Glycoalkaloids

Glycoalkaloids are the class of potentially toxic compounds. Typically potato tubers contain about 20-60 mg of total glycoalkaloid (TGA) per 100 g of freeze-dried

matter, equivalent to 4-12 mg of TGA per 100 g fresh weight. At these concentrations glycoalkaloids enhance potato flavour. However, at concentrations greater than 20 mg per 100 g they impart a bitter taste and can cause gastroenteritis symptoms, coma, and even death (Korpan et al., 2004). The toxic dose is considered to be approximately 2-5 mg/ kg body weight (bw) whereas the lethal dose is probably 3-6 mg/ kg (Langkilde et al., 2012). Due to human toxicity, 200 mg TGA/kg fw potatoes are accepted as the upper safety limit. The joint FAO/WHO Expert Committee on Food Additives (JECFA) is considered a TGA content of less than 100 mg/kg potatoes FW of no concern. These are potato-based recommendations. Presently available epidemiological and experimental data from human and laboratory animal studies are not sufficient to determine a realistic, safe level of intake (Mensinga et al., 2005). Several poisoning cases have been documented by the consumption of potatoes containing high levels of a-solanine and a-chaconine (Chen and Miller, 2001).

There are two main mechanisms of glycoalkaloid toxicity. First, GAs disrupts the cell membrane by causing the formation of destabilising complexes of the lipophilic moiety of the gas with cholesterol (Mensinga et al., 2005). Recent cell culture and experimental animal studies have demonstrated that GAs may adversely influence intestinal permeability. Cell lysis in the gastrointestinal tract can lead to abdominal cramps, diarrhoea, and eventually internal hemorrhaging (Gomah and Nenaah, 2011). With regard to membrane-disruptive activity, chacotriose-based glycoalkaloids are highly active compared to solatriose-based compounds (Roddick et al., 2001). Second, glycoalkaloids inhibit acetylcholinesterase (AChE, the enzyme responsible for nerve impulse transmission) and butyrylcholinesterase (an enzyme that is possibly protective against specific toxins) activities. The physiological effects of cholinesterase inhibition include sweating, vomiting, diarrhoea, and muscle spasms. Severe poisoning may cause serious adverse events, such as paralysis, respiratory insufficiency, cardiac failure, and coma. Cases of lethal poisoning have been reported at estimated doses greater than 3 mg TGA/kg bw (Smith et al., 2004).

It was stated that since the two GAs share the same steroidal aglycone (solasodine), this difference is presumably due to the different structures of the carbohydrate side chains. Therefore, the carbohydrate moiety has a significant role in

influencing cell membrane disruptions and embryotoxic/teratogenic effects of glycoalkaloids (Smith et al., 2004).

2.5.3 Beneficial Effects of Glycoalkaloids

Although glycoalkaloids are toxic compounds at certain levels, but they have some beneficial effects. In recent years, medicinal uses of glycoalkaloids has been a focus of scientific and pharmacological attention. For example, solamargine and solasodine exhibit potent cytotoxicity to human hepatoma cells (Hep3B) by apoptosis, which is the major process responsible for cell death in various physiological events (Ji et al., 2012). Solasodine, solamargine, and solasonine from *Solanum incanum* L. showed liver protective effects against CCl4-induced liver damage (Friedman et al., 2009). Furthermore, a-chaconine, a-solanine, a–solamargine, a-solasonine, a-tomatine (being the most effective), and some of their hydrolysis products inhibit the growth of human colon (HT29) and liver carcinoma (HepG2) cells (Lee et al., 2013). Plasma low-density lipoprotein cholesterol and triglycerides in hamsters is lowered by a- tomatine. The immune response is enhanced by a-tomatine inducing cytokines in immunized animals (Friedman, 2005). Solanine and chaconine either individually or as mixtures reduced the numbers of the cervical (HeLa), liver (HepG2), lymphoma (U937), stomach (AGS and KATO III) cancer cells (Friedman et al., 2009). Moreover, solamargine and solasonine isolated from *Solanum sodomaeum* have been utilized to treat malignant human skin tumours including basal and squamous cell carcinomas (Lee et al., 2013). Very recently a mixture of solamargine and solasonine has been developed to treat various cancer types such as glioblastoma multiform, colon rectal, bladder, liver, basal cell and squamous, metastasised melanoma to the lungs and other respiratory cancers, cell cancers. It was claimed that promising results obtained, e.g., reduction in tumour size and growth rates and, extension of life from treatments of more than 40 patients (Friedman et al., 2009).

Furthermore, solasodine present in solanaceae plants has gained significant importance globally. It can be converted to 16-dehydropregnenolone, a key intermediate in the synthesis of steroid drugs (Eltayeb et al., 1997). The leptines found in *Solanum chacoense Bitt*. are natural antifeedants to the Colorado potato beetle, *Leptinotarsa*

decemLineata, with the Leptine I displaying deterrent activity on adult feeding and inhibiting larval development (Sinden et al., 1986). Moderate resistance to the Colorado potato beetle in the hybrids between *S.chacoense* and *S.tuberosum* has been reported (Laurila, 2004). Solamargine, solasonine and tomatine inhibited larval growth of the red flour beetle, *Tribolum castaneum*. Tomatine also showed inhibitory activity on tobacco hornworm, *Manduca sexta* Johan (Clarke and Malpathak, 2005). A fraction of a mixture containing solamargine and solasonine mixture from the fruit of *S. mammosum* was shown to display a strong molluscicidal property on *Lymnaea cubensis* snails. Tomatine was reported to have antibacterial effects on gram positive bacteria that infect humans (Lee et al., 2013).

2.6 IDENTIFICATION TECHNIQUES

Spectroscopic identification of the organic compounds is important in structure elucidation. There are many identification methods such as ultraviolet/visible spectroscopy, infra-red spectroscopy, mass spectrometry and nuclear magnetic resonance spectroscopy. Spectroscopic method should be substituted the conventional method which takes a longer time in the identification of compounds. Spectroscopy is involving the interaction between the photons or electromagnetic wave with the matter. The spectroscopic data will be interpreted by using spectra and obtains the molecular structure from the information from this data support. Different types of interaction with matter (molecule) depend on the frequency and wavelength of the radiation.

2.7 STUDIED PLANTS

A number of plants selected from different families, mainly from Fabaceae, Solanaceae, Apiaceae, Zingiberaceae, Piperaceae, Rubiaceae and Olacaceae. Total 33 plants were studied of which three plants *Solanum Tubersum* (Potato), *Solanum melongena* (Brinjals) and *Capsicum annum* (Chillie), having the highest activity among all, are selected to isolate the potential compound for bioassay. The details of all the plants are mentioned in Table 2.2.

Table 2.2: Names of the plants selected for antioxidant and AChE activity

Plant Name	Common Name	Family	Parts used
Centilla asiatica	Centella	Apiaceae	Leaves
Piper sarmentosum	Wild pepper	Piperaceae	Leaves, shoots
Curcuma longa	Turmeric	Zingiberaceae	Rhizome
Coriandrum sativum	Coriander	Apiaceae	Leaves, see
Zingber officinale	Ginger root	Zingiberaceae	Leaves
Solanum melongena	Eggplant	Solanaceae	Non-edible parts
Citrus limon	Lemons	Rutaceae	Leaves, scales
Piper betle	Paan leaf	Moringaceae	Leaves
Moringa oleifera	Drumstick tree	Piperaceae	Leaves
Morinda citrifolia	Mulberry, Noni	Rubiaceae	Leaves
Solenostemon scutellarioides	Coleus	Lamiaceae	Leaves
Capsicum annuum	Pepper	Solanaceae	Non-edible parts,roots
Lantana camara	Lantanas	Verbenaceae	Leaves
Solanum torvum	Turkey berry	Solanaceae	Fruit
Amaranthus gangeticus	Chines spinach	Amaranthaceae	Leaves
Uncaria gambir		ubiaceae	Fruit
Aquilaria malaccensis	Agarwood	Thymelaeaceae	Leaves
Swietenia macrophylla	Bigleaf maghony	Meliaceae	Leaves
Solanum tuberosum	Potato	Solanaceae	Non-edible parts, roots
Gynandropsis gynandra	African cabbage	Capparidaceae	Leaves, flower
Mimosa pudica	Sensitive plant	Fabaceae	Leaves, seeds
Psophocarpus tetragonolobus	Wing bean	Fabaceae	Leaves, flower

Table 2.2 Continued

Crotalaria pumila	Rattlepod	Fabaceae	Seeds, Sprouts
Lablab purpureus	Saim bean	Fabaceae	Seeds, Sprouts
Phaseolus vulgaris	String bean	Fabaceae	Leaves, roots
Vigna radiata	Mung bean	Fabaceae	Seeds, Sprouts
Cicer arietinum	Chickpea	Fabaceae	Seeds, Sprouts
Vigna unguiculata	Cowpea	Fabaceae	Seeds, Sprouts
Trigonella	Fenugreek	Fabaceae	Seeds, Sprouts
Foenumgraecum	Bird's foot	Fabaceae	Seeds, Sprouts
Phaseolus vulgari		Fabaceae	Seeds, Sprouts
Scorodocarpus boroneenis	Kulim	Olacaceae	Leaves

The selection of plants was made on the basis of all the three approaches, i.e ethnobotinical, use in folk medicines and cited in the literature (Filho et al., 2006).

As we aimed to study the most common vegetables of the family solanaceae containing the alkaloidal compounds, that have good antioxidant and anti-actylcholinesterase activity as well. So, we selected the three common vegetables as these are commonly used vegetables.

2.7.1 *Solanum tuberosum* (Potato)

Potato belong to the family Solanaceae, genus *Solanum*, section *Petota*. It was divided into two subsections; subsection *Potatoe* containing both cultivated and wild (Drobyazina and Khavkin, 2011). The tuber is the edible part of the potato, which is a part of the stem that stores food and plays a role in propagation. The tuber is also regarded as an enlarged stolon. Stolons are formed from lateral buds at the bottom of the stem (Burlingame et al., 2009). It can be seen in Figure 2.10.

Despite their status as food products, potato tubers contain potentially hazardous glycoalkaloids (GAs), which are the result of an evolutionary selection process serve as defence compounds against herbivores and pathogens (Gemeinholzer and Wink, 2002; Wink, 2003). Major alkaloids in commercial cultivars are α-solanine and α-chaconine; together they account for 95% of total GAs in tubers. As Figure 2.2 illustrates, the two compounds consist of the aglycone solanidine, which has an nonpolar lipophilic steroid nucleus fused with two nitrogen-containing heterocyclic rings. The glycosides carry a polar, water-soluble trisaccharide moiety at C-3 (Lachman et al. 2001). This can consist of a glucose, galactose and rhamnose molecule as in α-solanine or two rhamnose molecules and a glucose molecule as in α-chaconine.

Potatoes are among the most widely-grown crop plants in the world, giving good yields under various soil and weather conditions (Drobyazina and Khavkin, 2011). Potato has been ranked as the fourth important food crop worldwide after wheat, rice and corn, and one of the main vegetables consumed in European diets (Tajner-Czopek et al., 2012). More recently, potato has been ranked third by the FAO. According to Lachman et al. (2001), annual worldwide production of potatoes is approximately 350 million tons (771,618 million lbs). The US potato production was about 44 billion lbs (0.02 billion tons) in 2006 (USDA, 2007). The world average per capita consumption in 2005 was estimated at 33.7 kg (74.3 lbs) (FAO, 2008), while the US per capita consumption of potatoes is about 57kg (126 lbs) (National Potato Council, 2008). Highest potato consumption is in Europe with a per capita consumption of about 96 kg, followed by North America at 57 kg. Per capita consumption is low in Africa and Latin America, but is increasing (FAO, 2008).

The high consumption rate of potatoes is attributed to both their palatability and high nutritive value (Rytel, 2012). Potatoes serves as a major food source, as well as an inexpensive source of energy and good quality protein (Lachman et al., 2001).

Figure 2.10: *Solanum tuberosum* plant with tuber and peel.

Source: Kakhia (2003)

2.7.2 *Solanum melongena* (Brinjals)

Solanum melongena L. commonly known as eggplant, aubergine, guinea squash or brinjal, is an economically important vegetable crop of tropical and temperate parts of the world. Eggplant fruits are quite high in nutritive value and can justifiably be compared with tomato as a good source of vitamins, dietary fibre and minerals (particularly iron) (Tiwari and Gambhhir, 2011). It has been used in traditional medicines. For example, tissue fractions have been used for treatment of asthma, bronchitis, cholera, and dysuria; fruits and leaves are beneficial in lowering blood cholesterol (Sinead et al., 2011). Its production is severely affected by biotic and abiotic stresses, as the levels of resistance to those factors are insufficient. There are many wild species of eggplant showing resistance to important pests that influence commercial eggplant production. Among the wild relatives, which can be exploited to increase genetic variability, *S. torvum* has been identified to carry the traits of resistance to most of the serious diseases affecting eggplant, particularly bacterial and fungal wilts, and nematodes. Interspecific hybrids between wild and cultivated species have been successful in only a few cases (Gousset et al., 2005). Brinjal plant and fruit are shown in Figure 2.11.

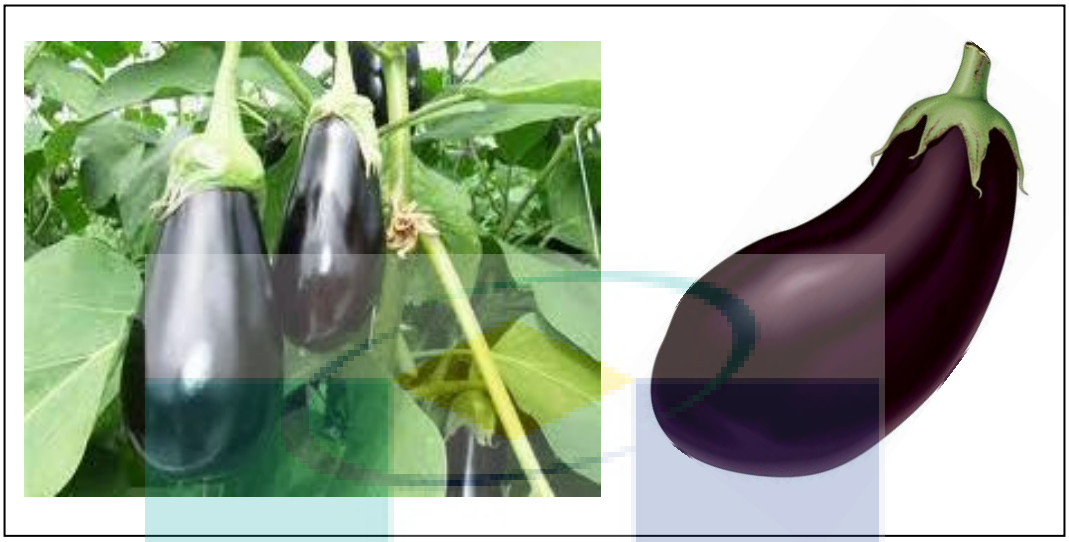


Figure 2.11: *Solanum melongena* plant and fruit.

Source: Kakhia (2003)

2.7.3 *Capsicum annuum* (Chilli pepper)

Chilli is widely used as a condiment and spice all over the world. It is commonly used in Malaysian cuisines. Chilli peppers are native to South and Central America, but adapted to the Malaysian tropical region. They were introduced in South Asia in the 1500s and have come to dominate the world spice trade. Chilli pepper is thought to be the most popular spice with over 20% of the worlds' population using it in some form or the other (Anwarul-Huq and Fatimah, 2010).

Pepper fruit (Figure 2.12) is popular vegetables because of the combination of colour, taste and nutrition. They are used as foods and spice. Moreover, the red pepper fruit has been used for many years as a source of pigments to add or change the colour of food stuffs. Fresh peppers are a good source of vitamin C and E as well as provitamin A and carotenoid compounds with well known antioxidant properties (Serrano-Mart ńez et al., 2008).

It is one of the important spices used very widely in culinary, pharmaceutical and beverage industries throughout the world. Chilli both in ripe and green fruit stage is an important condiment used for imparting pungency which is due to an active principle 'capsaicin' an alkaloid present in the pericarp and placenta. Capsaicin is a digestive stimulant and an important ingredient of daily diet. Chilli is a source of vitamin C (ascorbic acid) in food and beverage industries. It has acquired a great importance because of the presence of oleoresin, which permits better distribution of colour and flavour in the food. The heterosis and combining ability studies on quality parameters such as ascorbic acid content, sugar content and colour value, etc. are limited in Chilli (Nandadevi and Hosmani, 2003)

Figure 2.12: *Capsicum annuum* plant with fruit.

Source: Kakhia (2003)

2.8 BENEFICIAL EFFECTS OF SOLANUM SPECIES FOR HUMAN HEALTH

Since the early 1960s, the growth in potato production area has rapidly overtaken all other food crops in developing countries. It is a fundamental element in the food security for millions of people across South America, Africa, and Asia, including Central Asia (Keith, 2007).

Potato is the main source of low fat rich carbohydrates, with one-fourth calories of bread. Boiled potatoes have more protein and twice the calcium as compare to maiz. Potato along with the skin provides 10% of recommended daily intake of fibre. A medium boiled potato have sufficient amount of vitamin C, iron, potassium and zinc, which is required for an adult (Liu et al., 2012). The high vitamin C content enhances the iron absorption. Potato also contains vitamin B and trace elements like manganese, Chromium, Selenium and Molybdenum. Beside these elements, potato also contains the toxic components known as glycoalkaloids. It protects the plants against fungi and insects. It can be removed by cutting away the green parts of potato (Pandey, 2007).

Potatoes can have white, yellow, pink, red, purple, and even blue flesh colour. Yellow is primarily due to the presence of carotenoid concentrations, and the red, purple, and blue colour to anthocyanins. Both are antioxidants and believed to play an important role in preventing cancer and diseases related to ageing such as Alzheimer's and Parkinson.

2.9 CORRELATION OF ANTIOXIDANTS WITH ALZHEIMER'S DISEASE

The humans are constantly exposed to free radicals created by man made environment such as pollutants, cigarette smoke and electromagnetic radiations. Natural resources such as radon, cosmic radiation, as well as cellular metabolisms (respiratory burst, enzyme reactions) also add free radicals to the environment. The most common reported cellular free radicals are hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$) and nitric monoxide ($NO^{\cdot}$). Even some other molecules like hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are not free radicals; they are reported to generate free radicals through various chemical reactions in many cases (Gilgun and Melamed, 2001).

The human body produces oxygen free radicals and other reactive oxygen species as byproducts through numerous physiological and biochemical processes. Oxygen related free radicals (superoxide and hydroxyl radicals) and reactive species (hydrogen peroxide, nitric oxide, peroxynitrite and hypochlorous acid), are produced in the body, primarily as a result of aerobic metabolism (Halliwell and Cross, 1994, and Konat, 2003). At the same time, natural antioxidants, such as glutathione, arginine,

citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A and tea polyphenols help to regulate the ROS thus generated. Antioxidant is further supported with antioxidant enzymes, e.g. superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase thus exert synergistic actions in removing free radicals (Yun-Zhong et al., 2002).

Overproduction of free radicals can cause oxidative damage to biomolecules, (lipids, proteins, DNA), eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischaemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans (Uttara et al., 2009). Excess NO is cytotoxic either by combining with tyrosine that is essential for catalytic function of enzyme ribonucleoside diphosphate reductase or by forming $ONOO^-$. Excess vascular O_2 production could contribute to hypertension and vasospasm (Opazo et al., 2002).

ROS are particularly active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, whose metabolism is a factory of ROS, which are unique to the brain and serve as sources of oxidative stress. ROS attack glial cells and neurons, which are post-mitotic cells and therefore, they are particularly sensitive to free radicals, leading to neuronal damage (Gilgun and Melamed, 2006). It has been reported that the deleterious effects of ROS on human cells may end in oxidative injury leading to programmed cell death, i.e. apoptosis (Manda et al., 2009).

Ayasolla et al. (2004), reported inhibition of A-β induced NO- production by Vitamins E and C. Some antioxidants such as melatonin, curcumin and Ginkgo biloba are also reported to have a protective effect against A-β mediated neurotoxicity. In clinical trials of AD, some molecules having antioxidant properties such as Vitamin E and Ginkgo biloba fraction have shown modest benefit. The Ginkgo biloba fraction EGb 761 has shown neuroprotective effects in several animal models (Korolainen et al., 2002), to improve or maintain cognitive function in AD patients (Oken et al., 1998). Most of the effects of Ginkgo biloba of scavenging the free radicals and decline in AD effects, have been attributed to its antioxidant properties, probably due to flavonoid or terpenoid constituents in the fraction (Yao et al., 2001).

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter discusses the methods and materials used to carry out the whole analysis. Plant material, analysis methods and all the reagents and chemicals used for analyzing the samples to get the bio activities. Starting from fractionation, isolation of pure compound (alpha solanine), and its bioassays have been described step wise including the instruments used for this purpose.

3.2 MATERIALS AND CHEMICALS

All the chemical and reagents used in this study were obtained from Sigma Aldrich (St. Louis, U.S). HPLC-grade acetonitrile and methanol were purchasd from Sigma Aldrich, (St. Louis, U.S). Tris-HCl and ammonium dihydrogen phosphate (>99%) were purchased from Sigma Aldrich (St. Louis, U.S) and Merck (Darmstadt Germany), respectively. C18 reverse phase column was purchased from Agilent (Munster, Germany). Other organic solvent used for extraction and fractionation includes ethanol (Merck), methanol (Sigma Aldrich), chloroform (Merck), acetone (Merck), ethyl acetate (Merck) and toluene (Merck).

3.2.1 PLANT MATERIALS COLLECTION AND IDENTIFICATION

Plant samples were collected from different areas during September 2010 to January 2012. *Curcuma longa*, *Coriandrum sativum*, *Zingber officinale*, *Citrus limon*, *Piper betle*, *Amaranthus* gangeticus, and *Uncaria gambir* were collected from the fresh

vegetable market in Kuantan, Pahang. *Centilla asiatica, Piper sarmentosum, Morinda citrifolia, Solenostemon scutellarioides, Lantana camara, Swietenia macrophylla, Gynandropsis gynandra,* and *Mimosa pudica* were collected from the forest in the area of Seri Jaya, near UMP campus. *Moringa oleifera, Aquilaria malaccensis,* and *Scorodocarpus, boroneenis* were collected from the Forest Research Insititute Malaysia (FRIM), Kuala Lumpur. The beans *Psophocarpus tetragonolobus, Lablab purpureus, Crotalaria pumila, Phaseolus vulgaris, Vigna radiate, Trigonella Foenumgraecum,* and *Phaseolus vulgari* were purchased from the local hypermarket in Kuantan. While *Solanum tuberosum, Solanum melongena* and *Capsicum annuum* were grown in the UMP Gambang campus.

On the basis of DPPH and AChE activity, the non-edible part of three vegetables, namely *Solanum tuberosum, Solanum melongena,* and *Capsicum annuum* were selected for further study. The selected plants were grown in UMP Gambang campus and the samples were collected in April; 2012. The plants were identified by taxonomist Shamsul Khamis, (UPM). Voucher for all specimens were deposited at the Institute of Bioscience, University Putra Malaysia. Some tubers of the *S. tuberosum* were kept for budding and remaining were peeled off and use the waste (peels) as raw material. For *S. melongena* and *C. annuum,* the stalks by which it is hanging with the plant, used as raw material.

3.3 APPRATUS AND INSTRUMENTS

This section includes a brief detail of the instruments used in this study to carry out different experiments in the laboratory. Buchi Rotavapor R-II system (R-215) was used for rotary evaporation, together with Eyela A-1000 S vacuum pump and Buchi R-II Heating bath as unit components. HITACHI (CR 21 GIII) Cooling Centrifuge was used to carry out centrifugation of the extracts. Genesys 10S UV-VIS spectrophotometer was used for the spectrophotometric analysis. A 96-well microplate reader (Tecan Infinite 200 Pro) was used for the enzyme and antioxidant activity assay.

3.4 METHODOLOGY

3.4.1 Preparation of buffers

Four buffers were prepared for anti-AChE assay. Buffer A (50 mM Tris- HCl, pH 8), Buffer B (50 mM, pH 8, containing 0.1% bovine serum albumin), Buffer C (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$) and Buffer D (50 mM) NaH_2PO_4 in Na_2HPO_4, pH 7.6. The buffers were filtered through a 0.45-mm, polyamide filter (Sartorius, Germany). Mobile phase was prepared in acetonitrile-water (1:1 v/v) acidified to pH 2 with orthophosphoric acid and stored at 4 ^oC before use.

3.4.2. Preparation of Dragendorff's stock solution

Dragendorff's stock solution A was prepared by dissolving bismuth subnitrate (0.6 g) in 2 mL of concentrated HCl and 10 mL of water. Stock solution B was prepared by dissolving 6 g potassium iodide in water (10 mL). These stock solutions A, B and 7 mL of concentrated HCL were mixed together and then made up to 400 mL with water and it was stored in dark coloured bottle.

3.4.3 Thin Layer Chromatography

Thin layer chromatography (TLC) was performed by aluminum and glass sheets from Merck (Germany) precoated with silica gel 60 F_{254} (0.2 mm thickness) to separate and detect the compounds present in the crude samples and to check the purity of the isolated compound. The spots were visualized under UV light at 254 nm and 365 nm, after spraying with Dragendorff's reagent. The presence of alkaloid was detected by the appearance of orange spot on the plate. After spraying, the plate was kept for drying to see if any spot appears on it. The compounds were identified by measuring their R_f value.

3.4.4 High Pressure Liquid Chromatography (HPLC)

Agilent Technologies (1260 Infinity, G1311) high pressure liquid chromatography with photodiode array detection was used. UV detection at 205, 208, 254 and 280 nm

were done. Flow rates were typically 1 mL/min. Column temperature was adjusted to 60 ℃. Mobile phases (acetonitrile-0.05 M monobasic ammonium phosphate buffer (30:70 v/v), at pH 6.5) were prepared fresh, sonicated and filtered through a 0.45 mm polyamide filter. The injector loop was 20 µL on isocratic mode (Alt et al., 2005). Details are shown below in Table 3.1.

Table 3.1: Operating condition of HPLC for glycoalkaloids

Parameters	**HPLC Agilent 1260 series**
Stationary phase :	Octadecylsilyl silica gel for reversed (C18), Zorbax column SB-C18 5µm (250mm ×4.6 mm).
Mobile phase :	acetonitrile (A), Tris-HCl buffer (pH 6.5) (B)
Column temperature:	Ambient (23 ℃)
Pressure :	400 bar
Flow rate :	1 mL/mint
Injection volume :	20 µL
Detection wavelength:	254, 280 nm
Mode :	Isocratic

3.4.5 Spectroscopic Study

An infrared (IR) spectrum of pure compound in the liquid form was recorded on Perkin Elmer 100 FT-IR spectrophotometer, using KBr disk. NMR spectra were recorded on Bruker Avance 500 Spectrometer measured at 500 MHz. using Chloroform as a solvent. A mass spectrum was taken by Asea Cyte using Waters Acquity™ Ultra Performance Liquid Chromatography system, with Acquity PDA Detector (Version 1.40.1932). The pure compound was further analyzed on Acquity TM waters LC-MS (Acquity TM waters UPLC), as described in Table 3.2. Mass fragmentation and molecular weight were obtained from MS-MS spectrum (Vas and Vekey 2004).

Table 3.2: Operating condition of LC-MS for glycoalkaloids

Instruments		**Acquity TM waters**
Software	:	Waters MassLynes 4.1
Column specification		AQUITY UPLC BEH C18, 1.7 µm, 2.1 x 50 mm
Electron spray ionization		
Cappilary voltage	:	2.7 kv
Fractionion cone	:	4.0 mm
Source temperature	:	100 ^{O}C
Desolvation temperature	:	35 ^{O}C
Cone glass flow	:	30 L/h
Desolvation gas flow	:	700 L/h
		ms, sweep reading 3, reading/ replicate 3, number of replicates 3

3.5 SAMPLE PREPARATION

On the basis of DPPH and AChE activity, the non-edible part of three vegetables, namely *Solanum tuberosum*, *Solanum melongena*, and *Capsicum annuum* were selected for further study. About 500 g of each sample was taken for extraction of the phytochemicals. The *S. tuberosum* tuber was peeled off and the waste (peels) was used as raw material. For *S. melongena* and *C. annuum*, the stalks were used as raw material.

The samples were allowed to dry at room temperature for 5 days and finally in an oven at 55 ^{0}C for 3 h. Thereafter, they were ground for 5 min to powder (500 µm) using a standard laboratory blender (BagMixer® lab blender, Thermo Fisher Scientific, U.S) and the powder stored in a desiccator before fractionation.

3.6 EXTRACTION AND FRACTIONATION

A 500 g portion of each of the powdered samples was defatted with n-Hexane. The defatted sample was dried, fractioned with methanol-water (4:1) by sonication for 30 min and repeated for 3 cycles. The extracts were filtered, pooled together and concentrated by Rotary evaporator to one third volume to get a brown fraction (fraction B). Sulphuric acid was added to the concentrated fraction to lower the pH to 2. Chloroform was added and the mixture was shaken well. The chloroform portion was

separated by a separating funnel and evaporated until it makes a brownish gum residue (Fraction C). Ammonium hydroxide was used to basify the aqueous phase, to pH 10 and fractionated with chloroform-methanol (3:1) to get a dark brown gum of alkaloidal fraction (Fraction D) (Uhliq et al., 2014). The fractionation procedure is summarized in Figure 3.1.

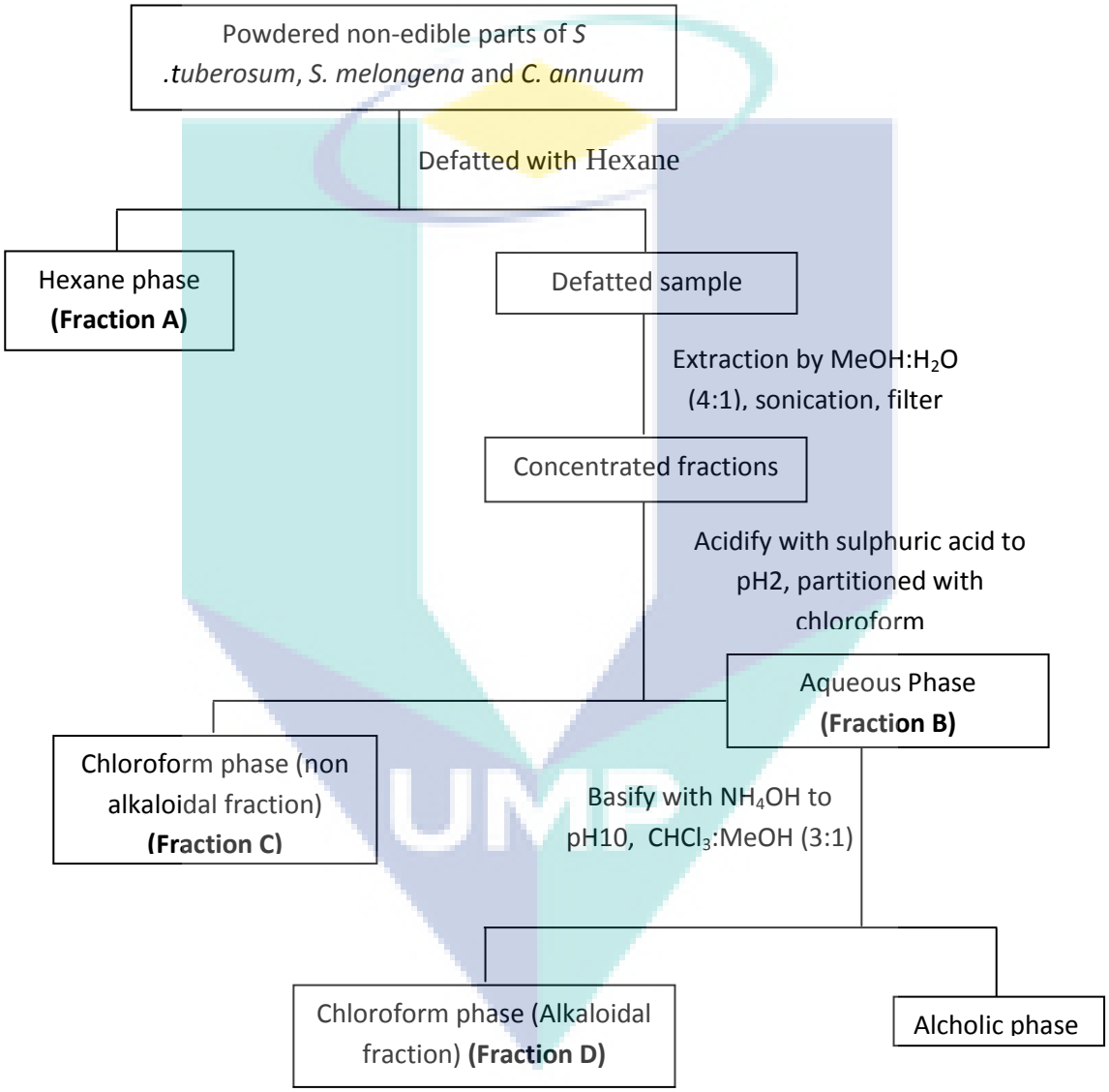


Figure 3.1: Fractionation of non-edible parts of *Solanum tuberosum*, *Solanum melongena* and *Capsicum annuum.*

3.7 DETERMINATION OF ANTIOXIDANT ACTIVITY

Two standard methods were used to assay antioxidant activity of the plants extracts. They include β-carotene bleaching assay and 1,2-diphenyl-2-picryl hydrazyl (DPPH) radical scavanging assay.

3.7.1 β-Carotene Bleaching Assay

Beta carotene bleaching assay was performed according to the method of Charoensiri et al. (2009). β-Carotene solution was prepared by dissolving 0.2 mg of beta carotene in 1 mL chloroform, in the round bottom flask containing 0.02 mL linoleic acid and 0.2 mL 100% Tween 20. The mixture was evaporated at 40 ℃ for 10 min using rotary evaporator to remove chloroform. Then 100 mL of distilled water was added with vigorous shaking to form an emulsion. Then 5 mL of the emulsion was taken out and transferred into test tubes containing 0.2 mL of sample. All test tubes were placed in a water bath at 45 ℃ for 2 h. The absorbance of samples was measured at 470 nm using spectrophotometer at the initial time (t=0) against a blank consisting of emulsion without β-carotene. A positive control (BHA) at a concentration (1 mg/mL) was used. The absorbance was recorded at 10, 20, 40, 70, 110 and 200 min. Bleaching capacity was calculated using the following formula:

$$I_B\% = \frac{S - A_{200}}{A_0 - A_{200}} \times 100 \qquad (3.1)$$

Where

I_B=Bleaching Inhibition

S = Sample absorbance at 120 min

A_o = Absorbance of control at zero min

A_{200} = Absorbance of control at 200 min

3.7.2 DPPH Free Radical Scavenging Assays

The radical scavenging activity of the extracts from 32 plant species under investigation was estimated according to the procedure modified by Braca et al. (2001). A 4 mL of 0.004% DPPH (dissolved in methanol) was mixed with 1 mL of plant extract of different concentrations (100, 200, 400, 600, 800 and 1000 µg/L). Butylated hdroxyanisole (BHA, 10-100 µg/mL) was used as reference standard. The mixture of 1 mL methanol and 1 mL DPPH solution was used as control. The mixture was vigorously shaken and kept at room temperature for 30 min in a dark place. The experiment was carried out in triplicates and absorbance was recorded at 517 nm by using UV-visible spectrophotometer (Genesys 10S). Radical scavenging activity was expressed as percent inhibition and was calculated using the following formula:

$$S_e(\%) = \frac{A_0 - A_i}{A_0} \times 100 \qquad (3.2)$$

Where

S_e = Scanvenging effect

A_o = Absorbance of control

A_i = Absorbance of sample

IC_{50} value (defined as the concentration causing 50% inhibition of absorbance) was determined from the plotted graph of scavenging activity against the concentrations of all the samples. IC_{50} was calculated using following formula:

$$Y = mx + c \qquad (3.3)$$

Where:

Y = % Inhibition

X = Concentration

C = Constant

m = Coefficient

3.8 DETERMINATION OF TOTAL PHENOLIC CONTENTS (TPC)

The total phenolic content was determined by spectrophotometric method described by Saeed et al. (2012). Briefly, 1 mL of sample (1 mg/mL) was mixed with 1 mL of Folin-Ciocalteu's phenol reagent. After 5 min, 10 mL of 7% sodium carbonate decahydrate solutions were added to the mixture, followed by the addition of 13 mL of deionized distilled water and mixed thoroughly. The mixture was kept in dark for 90 min at 23 ℃, then absorbance was recorded at 750 nm using UV-VIS spectrophotometer (Genesys 10S). The TPC was determined from extrapolation of calibration curve, which was made by preparing gallic acid solution (10-75 µg/mL). The estimation of phenolic compound was carried out in triplicate. The standard calibration curve was depicted in Appendix 4. The TPC was expressed as milligrams of gallic acid equivalent (GAE) per gram of dried sample, using following formula:

$$TPC = \frac{C \times V}{M} \tag{3.4}$$

Where:

TPC = Total phenolic content

C = Gallic acid (µg/mL) (i.e 0-200 µg/mL)

V = Volume of plant fraction (mL)

M = Sample weigh (mg)

3.9 DETERMINATION OF TOTAL FLAVANOID CONTENT (TFC)

The total flavonoid content of each of the specie was determined according to the method of Marinova et al. (2005) using aluminium chloride colorimetric assay. Briefly, 1 mL of appropriately diluted samples (1 mg/mL), standard solution of kaempferol, or blank (ethanol) were transferred into a 10 mL volumetric flask containing water (4 mL) and 5% $NaNO_2$ (0.3 mL). After 5 min, 0.3 mL of 10% (w/v) $AlCl_3$ solution was added with mixing. After 6 min, 2 mL of 1 M NaOH was added. The solution was then immediately diluted to a volume of 10 mL with water and mixed thoroughly. The sample was incubated at room temperature for 30 min. After incubation, the absorbance at 415 nm

was measured using a UV-Vis spectrophotometer. Samples were analyzed in triplicates. Flavonoid content was calculated based on the kaempferol calibration curve (100, 200, 400, 600, 800 and 1000 μg/mL) and the result were expressed as kaempferol equivalents (mg KE/g dry weight material).

3.10 ACETYLECHOLINESTERASE ASSAY BY MICROPLATE

Acetylcholinesterase activity assay was carried out by 96-well microplate method adopted by Ingkaninan et al. (2003) following the Ellman's method (Ellman et al., 1961). In this method, enzyme hydrolyzes the substrate, acetylthiocholine resulting in the formation of thiocholine which reacts with 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Sigma product no. D218200) to produce -nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which is detected at 412 nm.

Microplate wells were filled with 205 μL of reaction mixture. Of which 125 μL of Ellman's reagent (0.15 mM final concentration of 5, 5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH 7.4), 20 μL of acetylcholinesterase (final concentration 0.025 U/mL) in 0.1 M phosphate buffer pH 7.4), 20 μL of test compound solution in buffer, and 20 μL of buffer B. For negative controls, test solution was replaced by corresponding volume of ethanol. The enzymatic reaction was initiated by the addition of 20 μL of acetylthiocholine iodide (ATCI) in distilled water. The plate was shaken for 2 S and increase in absorbance was monitored at 412 nm at 30 ℃ for 10 min. Physiostigmin was used as the positive control. All assays were carried out in triplicate in 96-well microplate reader (Tecan Infinite 200 Pro). The % inhibition was calculated by the following formula:

$$I\% = \frac{A_s - A_i}{A_s} \times 100 \qquad (3.5)$$

Where

I% = Percent inhibition

A_s = Activity of enzyme without test compound

A_i = Activity of enzyme with test compound

The IC_{50} values were calculated by plotting graphs of the percentage inhibition against extract concentration.

3.11 TLC BIOAUTOGRAPHIC ASSAY

Thin layer chromatography is one of the most important and sensitive technique for the detection of different compounds, and it can guide for further steps of separation for a specific compound or class of compounds. TLC was used for qualitative studies of the anti-AChE and DPPH antioxidant activity of the alkaloids present in plant samples.

3.11.1 TLC Bioautography of anti- AChE activity

Anti-AChE activity by bioautography procedure recently reported by Zhongduo et al. (2009) was used for this bioassay. Briefly, 500 U of acetylcholinesterase V (EC3.1.1.7, Sigma, product no. C2888), was dissolved in 500 mL of 0.05 M Tris-HCl buffer, (pH 7.8) and stabilized by the addition of 0.1% bovine serum albumin (Sigma, product no. A4503). TLC plates were spotted with the extracts and the isolated compound. Galantamine (Sigma, product no G1620) was used as positive control. The separation was done by using chloroform and methanol (6:4) as the solvent system. Then the plate was dried absolutely with a blower. The plate was subsequently sprayed with enzyme and 1-nephthyl acetate solution followed by blowing quickly with a cold wind from a dryer until there was no flowing liquid on its surface. The plate was not dried absolutely to prevent inactivation of the enzyme. The plate was kept in a close vessel containing a small amount of water at 37 ℃ for 20 min to maintain humid environment without any contact with water. This allowed the enzyme to react with 1-nephthyl acetate completely. Then the solution of fast blue B salt was sprayed on TLC plate, the inhibited AChE spots appeared white while other parts purple.

In this assay the enzyme converts the 1-naphthyl acetate into naphthol, which reacts with Fast Blue B salt to make purple-colored background on TLC plates. 1-naphthol, reacts with one diazoamino group of Fast Blue B salt only to produce an unstable purple azo-product. The inhibitors of the enzyme prevent 1-naphthol formation which results in no purple coloration. The inhibitors of AChE in the samples produce white spots on the purple background.

3.11.2 TLC Bioautography for DPPH

The method was based on Gu et al. (2009) experiment. The extracts were subjected to TLC on silica gel (Merck) using the developing solvent system n-hexane-toluene-ethyl acetate-formic acid (2:5:2.5:0.5). The developed plates were allowed to stand in a fume hood until the solvents evaporated off. The plates were then sprayed with 2.0 mM DPPH solutions in methanol, when bands or spots with radical scavenging (antioxidant) activity immediately gave white and sometimes yellow colours on a purple background.

3.12 STATISTICAL ANALYSIS

The mean values of the data were analyzed using one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) version 17.0. P-value of less than 0.05 was considered significant. Linear Regression was used to correlate between TPC and bioacrttivities.

3.13 METHODOLOGY FLOW CHART

The methods and processes used in the study are shown in the methodology flow chart in Figure 3.2.

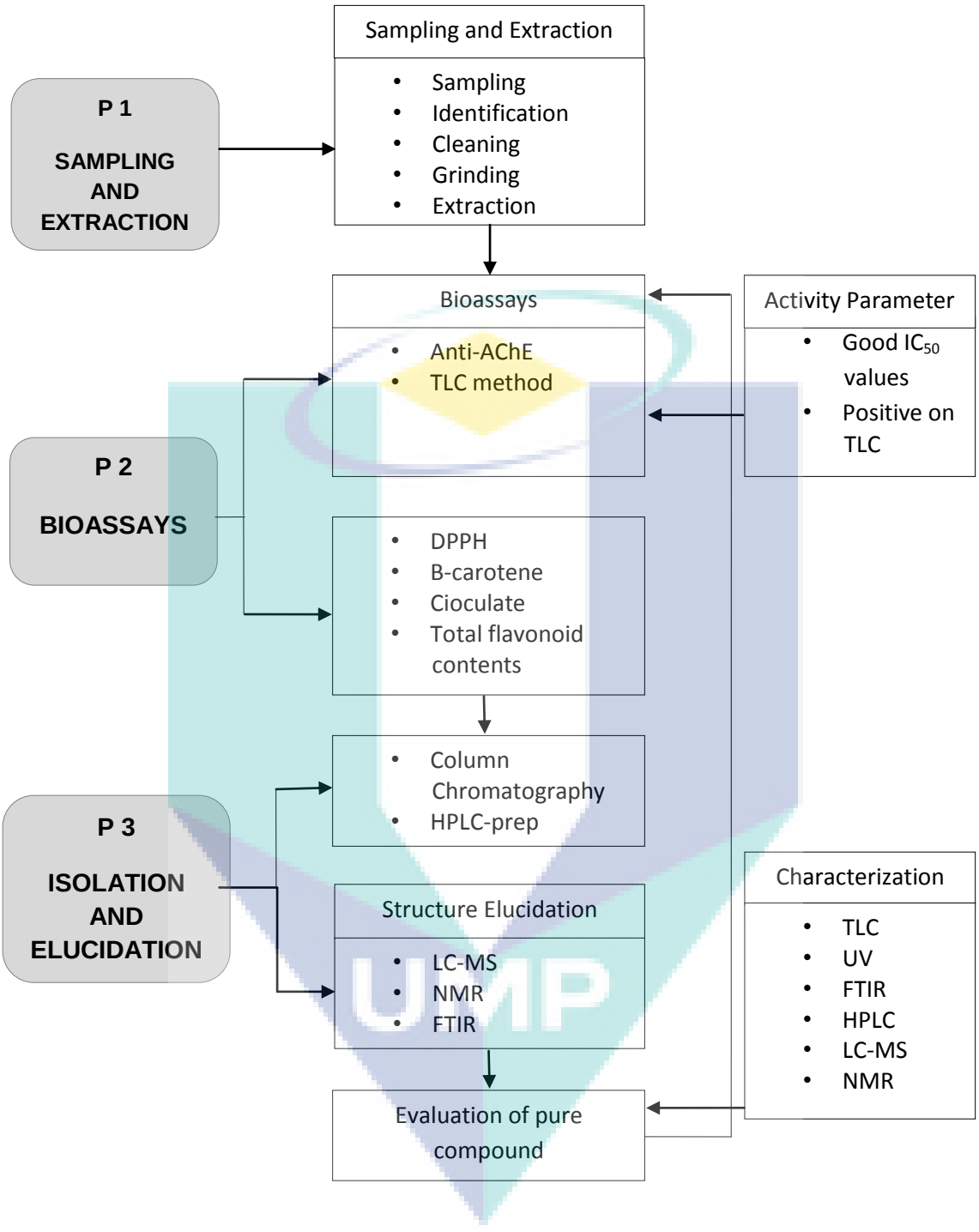


Figure 3.2: Methodology flow chart of experimental work.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

This chapter include the results obtained through the various analyses during this study. It starts with the initial screening of 32 plants based on their antioxidant and anti-acetylcholinesterase activity. Three most active plants were selected from 32 plants based on screening results and the alkaloidal compound was isolated from their non-edible parts. The potential of the crude extract and the isolated compound were evaluated for their bioactivity. Correlation of the TPC with the bioactivities were studied. Finally the structure of the isolated compound was determined by different spectroscopic methods.

4.2 SCREENING OF PLANTS FOR BIOLOGICAL ACTIVITIES

The extracts of 32 plant species belonging to different families were analyzed for their inhibitory activity against AChE enzyme and antioxidant activity, to get the potential compound on the basis of bioassay guided fractionation. The activity of screened plants is shown in Table 4.1.

Piper sarmentosum showed good antioxidant activity (58.23%) and medium anti-acetylcholinesterase activity (22.34%). It is worth mentionable here that *Piper sarmentosum* was analyzed for the first time for acetylcholinesterase activity in this study. The results are close to those reported by Vinuth et al. (2007), who used different solvents and got better results. It is probably because the fruit contains more active compounds as

compared to leaves. This plant has been used by elderly people as a folk medicine in Malaysia for mental health. Positive results on AChE inhibition assay supported its use as a memory enhancer.

Curcuma longa showed a medium antioxidant (34.48 %) and anti-AChE activity (31.44 %). These results are in agreement with that obtained by Shrikant and Kalpana (2008). This plant is used as a constituent of different foods and is believed to enhance the memory. The anti-AChE results obtained from this study supports its memory improving activity.

Coriandrum sativum leaves showed medium bioactivities for both antioxidant and anti-AChE assays (13.24 %, and 9.75%, respectively), while seed extract showed lower activities (8.29 %, and 5.38%). The results comply with the report of Panjwani et al. (2010), who studied the bioactivites of *C. Sativum* leaves. Their results indicate that leaves contain more active compounds compared to seeds. Leaves and seeds are important constituent of cooked foods. Besides its use as a flavour, it is also nutritionally important.

The last ten plants in Table 4.1 belong to family Fabaceae. Most of them are pulses, used as routine diet. The pulses have medium antioxidant activity, the lowest with *Lablab purpueus* (11.17%) and the highest with *Phaseolus vulgari* B (39.38%). These findings are in agreement with the results obtained by Lu & Foo (2000). Although these pulses were studied for their antioxidant activity, anti-AChE activity was not studied. On the basis of correlation between anti-AChE activity and antioxidant activity, the values of anti-AChE were calculated to be 9.37 and 30.47%, respectively.

The plants that possess highest activities for both bioassays, belongs to solanaceae family. Three solanum species *S. tuberosum, S. melogena* and *C. annuum* showed promising results against both bioassays. On the basis of preliminary results, solanum species were selected for thorough study.

Table 4.1: DPPH radical scavenging and anti-AChE activity of 32 studied plants.

Plant Name	Common Name	Parts used	DPPH scavenging (%)	Anti-AChE activity (%)
Centilla asiatica	Centella	Leaves	51.61 ±8.04	19.72 ±9.43
Piper sarmentosum	Wild pepper	Leaves	58.23 ±9.03	22.34 ±8.24
Curcuma longa	Tumeric	Rhizome	34.48 ±11.21	31.44 ±9.91
Coriandrum sativum	Coriander	Leaves	13.24 ±8.23	9.75 ±8.59
		Seed	8.29 ±9.77	5.38 ±7.16
Zingiber officinale	Ginger root	Leaves	8.29 ±7.54	31.44 ±9.3
Solanum melongena	Eggplant	Non-edible parts	62.37 ±11.25	58.56 ±12.45
		Leaves	54.21 ±10.38	51.72 ±10.04
Citrus limon	Lemons	Non-edible parts	14.19 ±7.82	9.78 ±7.35
Piper betle	Paan leaf	Leaves	15.25 ±9.12	12.32 ±8.32
Moringa oleifera	Drumstick tree	Leaves	28.72 ±10.41	25.42 ±9.63
Morinda citrifolia	Mulberry, Noni	Leaves	23.38 ±9.33	19.31 ±8.52
Solenostemon scutellarioides	Coleus	Leaves	15.93 ±8.34	13.26 ±9.62
Capsicum annuum	Pepper	Non-edible Parts	27.53 ±10.47	52.31 ±11.15
		Roots	21.44 ±10.73	27.43 ±10.23
Lantana camara	Lantanas	Leaves	27.50 ±11.14	23.82 ±9.24
Solanum torvum	Turkey berry	Fruit	37.61 ±10.25	33.65 ±10.43
Amaranthus gangeticus	Chines spinach	Leaves	12.31 ±8.92	7.25 ±5.42
Uncaria gambir	Ourouparia	Fruit	14.19 ±9.23	12.42 ±8.22
Aquilaria malaccensis	Agarwood	Leaves	6.71 ±7.81	3.23 ±6.31
Swietenia macrophylla	Bigleaf maghony	Scales	11.47 ±8.32	6.38 ±6.12
Solanum tuberosum	Potato	Non-edible parts	69.57 ±13.75	61.34 ±12.66
		Roots	56.28 ±12.34	52.68 ±10.55

Table 4.1 Continued

Gynandropsis gynandra	African cabbage	Leaves Flower	12.38 ±7.33 13.14 ±7.52	8.32 ±6.46 9.94 ±5.22
Mimosa pudica	Sensitive plant	Leaves Roots	25.31 ±8.44 38.56 ±9.75	21.27 ±7.43 32.29 ±8.34
Psophocarpus tetragonolobus	Wing bean	Leaves Seeds	12.19 ±6.62 13.24 ±0.53	8.62 ±6.61 6.41 ±5.32
Crotalaria pumila	Rattlepod	Leaves Flower	17.42 ±8.43 14.12 ±6.32	13.12 ±7.14 9.37 ±6.22
Lablab purpureus	Saim bean	Seeds	11.17 ±6.24	9.43 ±6.52
Phaseolus vulgaris (A)	String bean	Seeds	22.37 ±8.15	17.31 ±7.43
Vigna radiata	Mung bean	Seeds Sprouts	33.25 ±10.96 34.62 ±10.85	25.23 ±9.44 26.86 ±8.15
Cicer arietinum	Chickpea	Seeds Sprouts	32.91 ±9.84 33.32 ±9.75	23.96 ±7.23 26.82 ±7.34
Vigna unguiculata	Cowpea	Seeds	25.21 ±8.63	18.33 ±6.42
Trigonella	Fenugreek	Seeds Sprouts	22.32 ±7.54 24.43 ±7.45	16.75 ±6.53 20.13 ±7.63
Foenumgraecum	Bird's foot	Seeds	25.72 ±7.34	21.29 ±8.74
Phaseolus vulgari (B)		Seeds	39.38 ±11.26	30.47 ±9.85
Scorodocarpus boroneenis	Kulim	Leaves	4.87 ±5.12	1.62 ±5.91

*R = Red bean, B = Black bean

4.3 ANTIOXIDANT ACTIVITY OF THREE SELECTED PLANTS

Once the selected 32 plants were primarily screened for their antioxidants and anti-AChE activity, three of them were selected for thorough study of their bioactivity and for isolation of bioactive compounds. This time two standard assays methods were used for antioxidant activity. The methods included β-carotene bleaching assay and DPPH radical scavenging activity.

4.3.1 β–Carotene Bleaching Assay

According to Wang et al. (2009), the mechanism of beta-carotene bleaching assay is a free radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid by aerobic oxidation. The antioxidant activity of carotenoids is based on the formation of radical adducts of carotenoids with free radicals formed from linoleic acid. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups that attacks the highly unsaturated beta-carotene molecules. As beta-carotene molecules lose their double bonds by oxidation in this model system, in the absence of an antioxidant, the compound loses its chromophore and characteristic orange colour, which can be spectrophotometrically monitored (Wang et al., 2009).

Figure 4.1 shows the effect of addition of extract and the purified compound from 3 solanaceous species on the progress of β-carotene-bleaching. Spectrophotometric absorption values of different reaction mixtures from zero time up to 120 min were recorded and the degree of bleaching of β-carotene was observed at 20 min intervals. The values were expressed as a percentage of that observed at zero time. The values indicate the remaining amount of β-carotene, reciprocal to oxidation inhibition. It is clear from Fig 4.1 that *S.tuberosum* fractions possessed strong antioxidant activity compared to other two fractions and the pure compound. After 120 min, the unoxidized state of β-carotene was still maintained at 90% of that observed in zero time.

The other two solanaceous species had medium antioxidant activities, or perhaps acted as oxidants, during most of the assay period. In a study carried out by Ismail et al. (2004) for total antioxidant activity using β-carotene method for different vegetables. The total antioxidant activity of vegetables was more than non-edible parts of solanum species. The difference in the results of this study may be due to the difference of plant organs used, as the diffrent parts of plants has different ratio of the compounds. Furthermore, different extraction method also affect the results.

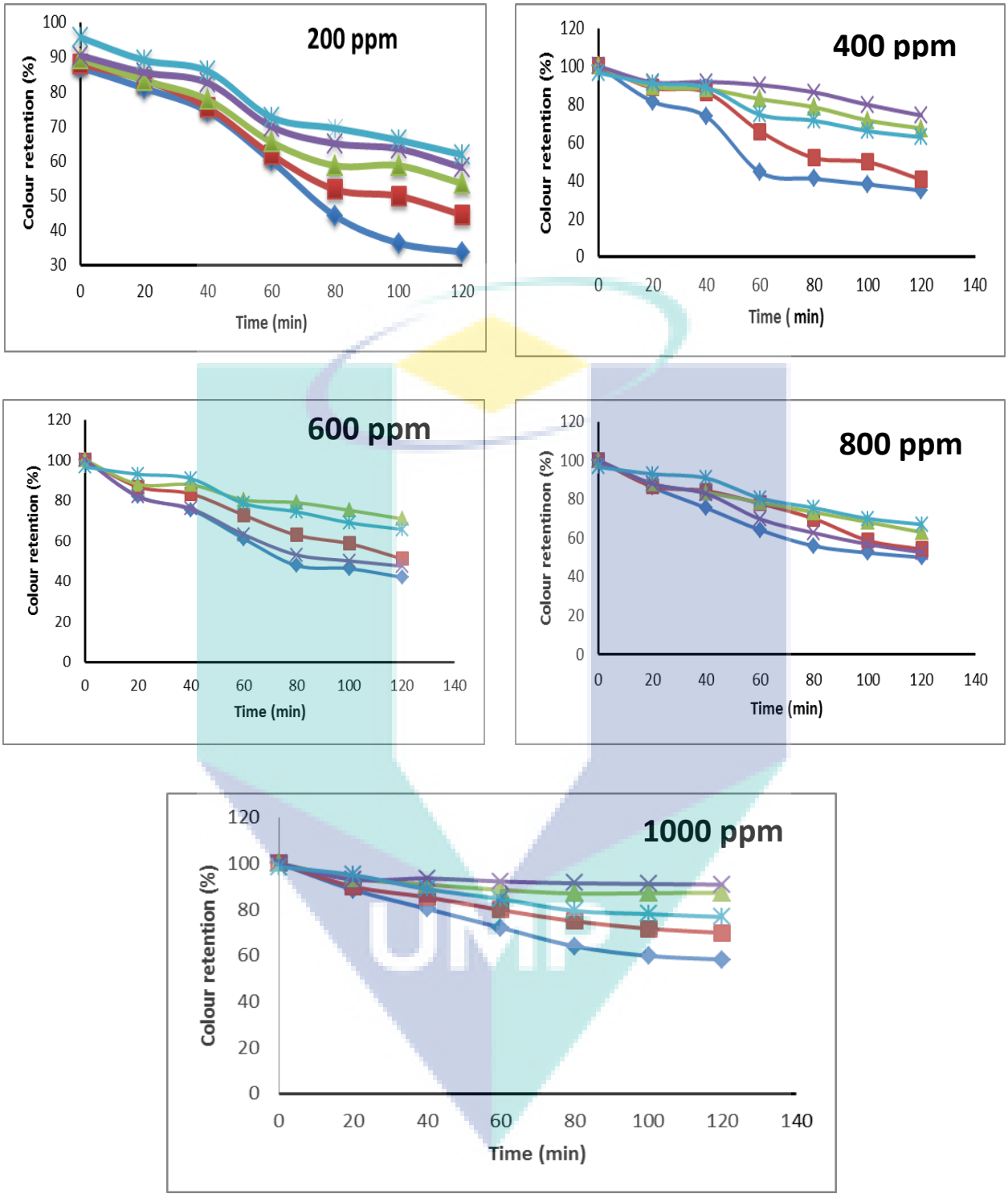


Figure 4.1: Mean total antioxidant activity of 3 plant extracts, isolated compound and positive control BHA, measured by using β-carotene bleaching assay.The symbols used in the Figures are: (▲) for *S.tuberosum*, (■) for *S.melongena*, and (▼) for *C.annuum.*

All studied plants possessed anti-oxidant properties as measured by β-carotene bleaching method. The highest antioxidant capacity was demonstrated by *S.tuberosum* fraction at 1000 ppm followed by the purified compound, and BHA, while *C.annuum* showed the lowest antioxidant activity. The highest activity of crude from *S.tuberosum* might be attributed to the synergistic effect of some other antioxidant species present in the crude extract.

4.3.2 DPPH Radical Scavenging Activity Inhibition (%) and IC_{50}

With the increasing interest in the function and diversity of antioxidants, some methods have been developed in order to determine this activity for plant extract. Among chemical methods applied to determine the antioxidant activity of a compound, DPPH is one of the most frequently used methods because it is practical, fast and stable. DPPH is a stable free radical compound that was used to test the scavenging ability of various samples in this study.

The results of the scavenging effect of fractions of solanaceous vegetables on DPPH radical are shown in Figure 4.2. The test samples were able to reduce the DPPH to the yellow-coloured diphenylpicrylhydrazine. There was a gradual increase of percent inhibition with increasing concentration of the extracts. The highest inhibition value among plant sample was demonstrated by *S. tuberosum* followed by the isolated compound, and *S. Melongena,* while *C. annuum* exhibited the lowest scavenging activity.

It was observed that the percent DPPH radical scavenging activity of *S. tuberosum* was higher compared to those of the rest of the species in the decreasing order of *S. tuberosum* > *S. melongena* > *C. annum,* respectively, while BHA was found to be the highest. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability as reported by Yazdanparast and Ardestani (2007). Bhat at el. (2013) studied the antioxidant activity of five leafy Malaysian vegetables and observed the similar trend.

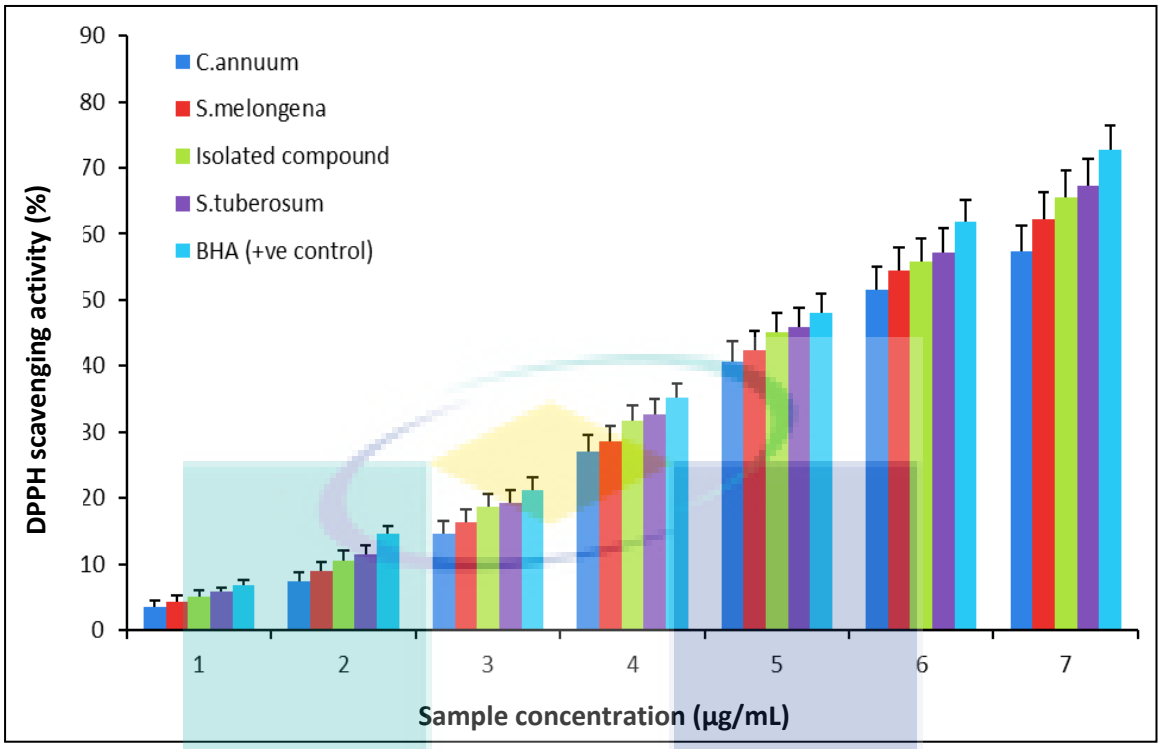


Figure 4.2: Scavenging activity of *S.tuberosum*, *S.melongena*, *C.annuum* and isolated compound, α-solanine, using BHA as positive control.

As shown in Table 4.2, IC_{50} of *S. tuberosum* was the lowest (679.8 µg/mL), followed by pure compound (708.37 µg/mL), *S. melongena* (745.34 µg/mL), and *C. annuum* (803.83 µg/mL). According to the results obtained, IC_{50} values of the synthetic antioxidant BHA was found the lowest (611.1 µg/mL). These results are in agreement with Mohdaly et al. (2010), who analyzed the potato peel and sugar pulp to study the antioxidant activities. While Seow et al. (2012), study the sweet potato plant which showed higher IC_{50} values than for non-edible parts of solanaceous plants. Xiuhong et al. (2012), who studied 20 different breeds of potato to analyze the phytochemical contents. The results are almost similar as that of non-edible parts of solanum species. Slight differences in the results are probably because of different sources of the sample. As the soil composition can be a factor, it plays an important role for the plant contents grown there. The statistical analysis is shown in Appendix 2.

Table 4.2: DPPH radical scavenging activity (% inhibition and IC_{50}) of solanum species.

Solanum species	Inhibition (%) [a]	IC_{50} (µg/mL) [b]
BHA	74.98 ±10.45	611.1 ±9.91
S.tuberosum	67.57 ±9.53	679.80 ±11.82
α-solanine	65.95 ±9.64	708.37 ±12.75
S.melongena	62.57 ±8.73	745.34 ±10.64
C.annuum	57.56 ±9.84	803.83 ±8.56

[a]Percent of inhibition and scavenging activity (µg mL^{-1}) as a mean of triplicate experiments.

[b] Values obtained from the regression lines with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum inhibition.

4.4 TOTAL PHENOLIC CONTENTS (TPC)

The TPC of three selected plants was determined by the Folin-Ciocalteu method at 517 nm using gallic acid as standard. The amount of total polyphenol was calculated from regression equation y = 0.0004x + 0.0513 obtained from the standard curve of galic acid. The results are presented in Table 4.3.

Among three plant samples, *S. tuberosum* showed the highest activity (128.82 ± 0.05) followed by *S. melongena* (114.81 ± 0.03) and *C. annuum* (111.94 ± 0.04). Helmja et al. (2007) analyzed the total phenolic contents and antioxidant activities of the same solanum vegetables species. They reported the highest TPC value in *S. melongena* skin and the lowest TPC in *C. annuum*. The lowest TPC in *C. annuum* in this study agrees with that reported by Helmja et al. (2007). The variation in the TPC content in *S. tuberosum* and *S. melongena* might be due to the difference in extraction method. In this study soxhlet extraction and maceration was used followed by fractionation, while they used ultrasonic extraction.

Juang et al., (2004) reported that the plants with highest total phenolic contents usually possess gallic, ellagic, caffeic, neochlorogenic, neochebulinic, chebulagic, chebulinic, ferulic, and chebulic acid, casuarinin, chebulanin, corilagin, terchebulin, punicalagin,1,6-di-O-galloyl-β-D-glucose, 3,4,6-tri-O-galloyl-β-D-glucose, 1,2,3,4,6-penta-O-galloyl-β-D-glucose, which are thought to be responsible for their high antioxidant activity. However, it should also be noted that some chemical group of ascorbic acid, organic acids, sugars, and aromatic amines can also react with Folin Ciocelteau reagent (Meda et al., 2005). Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu (FC) reagent (Prasad et al., 2009).

Table 4.3: Total phenolic ontents (TPC) of solanum species extract.

Solanum species	**TPC (mg GAE / g)**
S. tuberosum	128.82 ± 11.45
S. melongena	114.81 ± 10.53
C. annuum	111.94 ± 10.64

4.5 TOTAL FLAVONOID COMPOUNDS (TFC)

The TFC of three selected plants was determined by the method of Marinova et al. (2005) using the Kaempferol as standard and the absorbance was recorded at 435 nm. The amount of total flavonoid was calculated from regression equation y = 0.0005x + 0.0313 obtained from the standard curve of Kaempferol. The results are demonstrated in Table 4.4.

TFC in three selected plants varied from 155.56 to 85.56 mg KE/g of dry weight of sample. The *S. tuberosum* contained the highest average of TFC followed by *S. melongena* and the lowest content was found in *C. annuum*. Olajire and Azeez (2011) carried out a study on total flavonoid and total phenolic contents of several solanum species. It showed higher total flavonoid content for one species while for the rest of the species got lower flavonoid contents. The difference in the results may be due to the

difference in plant parts used. Other factors that might be responsible include climate, growth, temperature and storage conditions.

Table 4.4: Total flavonoid content (TFC) of solanum species extract.

Solanum species	**TFC (mg KE / g)**
S.tubersum	155.56 ± 9.55
S.melongena	146.36 ± 8.43
C.annuum	127.68 ± 10.37

It is evident from the study that solanaceous plants are a good source of phenlics and flavonoids that might contribute to its potency as memory enhancer. Since, *S. tuberosum* shows the highest phenol and flavonoids contents, it showed high antioxidant activity as well.

4.6 CORRELATION OF DPPH SCAVENGING ACTIVITY WITH TPC AND TFC

Because of the complex relationship between TPC and antioxidant capacities it is claim that (Yu et al., 2003) there is no correlation between the total phenolic content and the radical scavenging capacity. So it was important to examine the correlation between the TPC, TFC and antioxidant capacity (IC_{50}) of the extracts from selected plant species. Figure 4.3 shows the correlation between antioxidant capacity with TPC and TFC of solanum species. The data reveal that the TPC and TFC are significantly correlated with antioxidant capacity (R^2= 0.9706).

Several reports showed a close relationship between TPC and TFC with antioxidant activity. Tabart et al. (2006) investigated the same correlation between antioxidant activity with TPC & TFC of black currant leaf. Ehala et al. (2005) also reported the same correlation of bud extract of different berries. These results also agree with several other studies (Kähkönen et al., 1999; Shahidi and Naczk, 1995; Conforti et al., 2009).

This good correlation between TPC and TFC with DPPH scavenging activity indicates that the fractions obtained from solanum species have remarkable antioxidant activities. It was suggested that the composition of phenolic and flavonoids compounds is a key determinant of the radical scavenging activity. This is also implying that the antioxidant activity of extracts depends on the numbers and positions of the hydroxyl groups in relation to the glycosyl group and due to the presence of polyphenolics, carbonyl compounds. However, it can also be stated that the scavenging effect of fractions is not limited to phenolic and flavonoid compounds. The correlation of IC_{50} and TFC shows significant relation as compared to TPC.

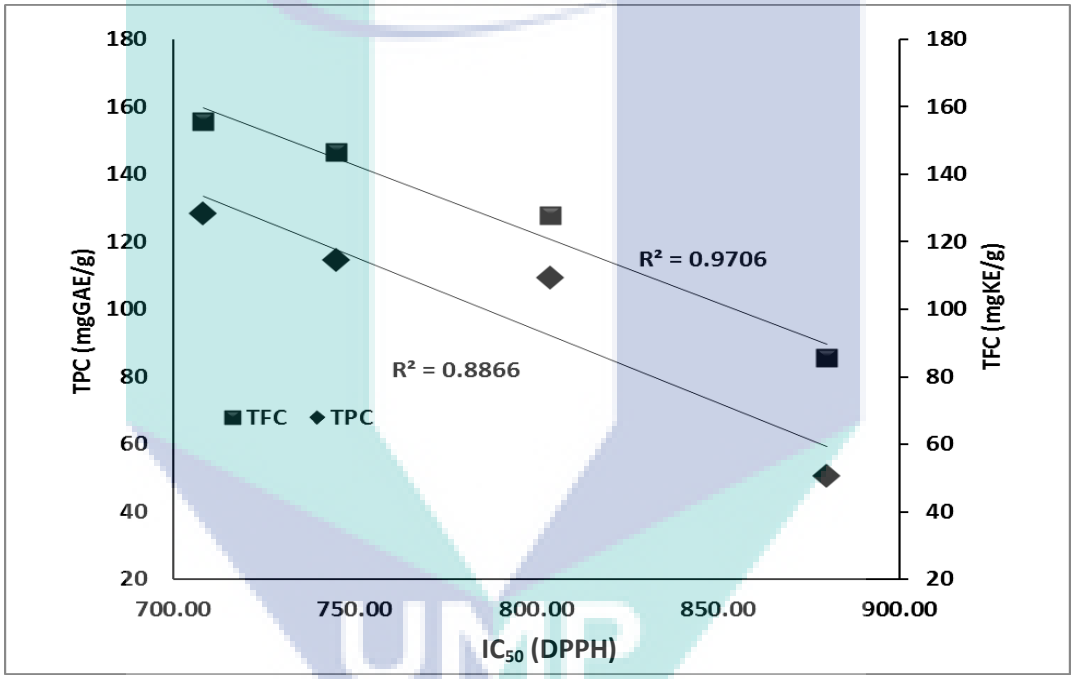


Figure 4.3: Corelation of antioxidant capacity (IC_{50}) of 3 plant extracts and standard BHA with TPC and TFC.

Although the correlation between antioxidant capacity and TFC of extracts was statistically significant, (Figure 4.3; R^2 = 0.9706), the correlation between TPC and antioxidant were stastistically less significant (R^2 = 0.8866). This indicates that the antioxidant capacity of the fraction is mostly due to its higher amount of flavonoids constituents. These results are in accordance with other reports in the literature, which showed strong positive correlation between IC_{50} and TFC. Rohman et al. (2010), reported a good correlation of TPC in red fruit with antioxidant activity. However, some others (Imeh and Khokhar, 2002; Bimal et al., 2013) repoted weak correlations of TPC with

antioxidant activity. The correlation coefficient between assays and α-solanine is shown in Table 4.5.

Table 4.5: Correlation coefficient between assays for different solanum species

Assay	AChE1C_{50}	DPPH1C_{50}	TPC
DPPHIC_{50}	0.973		
TPC	-0.756	-0.897	
TFC	-0.881	-0.973	0.898

4.7 ANTIACETYLCHOLINESTERASE ACTIVITY BY MICROPLATE

Through colorimetric method, results were expressed as IC_{50} values of AChE inhiibition. The value of IC_{50} of fractions was calculated from the regression equations of absorbance versus concentration of each sample. Figure 4.4 shows the anti-acetylcholinesterase activity of three solanum species along with isolated compound. All the three solanum species showed dose-dependent inhibition, i.e. the inhibition of acetylcholinesterase increase with the increase in the amount of plant sample that is acting as an inhibitor. Among three samples, *S. tuberosum* showed the highest value of inhibition (IC_{50} 689.9 µg/mL) followed by isolated compound (IC_{50} 725.70 µg/mL), *S. melongena* (IC_{50} 731.99 µg/mL), *C. annuum* (IC_{50} 851µg/mL). Lower the IC_{50} value, higher will be the inhibition activity. Physostigmine was used as standard and its activity (IC_{50} 670.24 µg/mL) was bit higher than *S.tuberosum* activity.

Dominik and Kamila (2012) studied 17 fruits and vegetables methanolic extracts for anti-AChE activity. Beside other samples, *S. tuberosum* juice sample showed higher activity (91% inhibition) which is higher than the non edible parts. Probably it is because the tuber of the potato contains more alkaloidal compounds as compare to the non-edible parts. Gholamhoseinian et al. (2009) studied anti-AChE activity on 100 different plants using methanol as solvent. The plants from the solanaceae family showed low inhibition (3.5 %) using the aerial parts. It indicates that the the aerial parts and flowers contains less

aklakoidal compounds that are responsible for the inhibition of AChE. Sanda et al. (2014) used the aerial parts of 26 different plants for the inhibition of AChE. All the plants showed the medium to higher anti-AChE activity.

Interestingly the fraction of *S. tuberosum* inhibited the enzyme more than the isolated pure compound. It is an alkaloidal compound, and most of the acetylcholinesterase inhibitors are alkaloidal compounds. The higher activity of crude compared to the pure compound might be due the synergistic effect of some other compound present in the crude extract. On whole, it can be postulated that *S. tuberosum* and *S. melongena* have strong AChE inhibitory effect. The ANOVA for anti-AChE is shown in appendix 11.

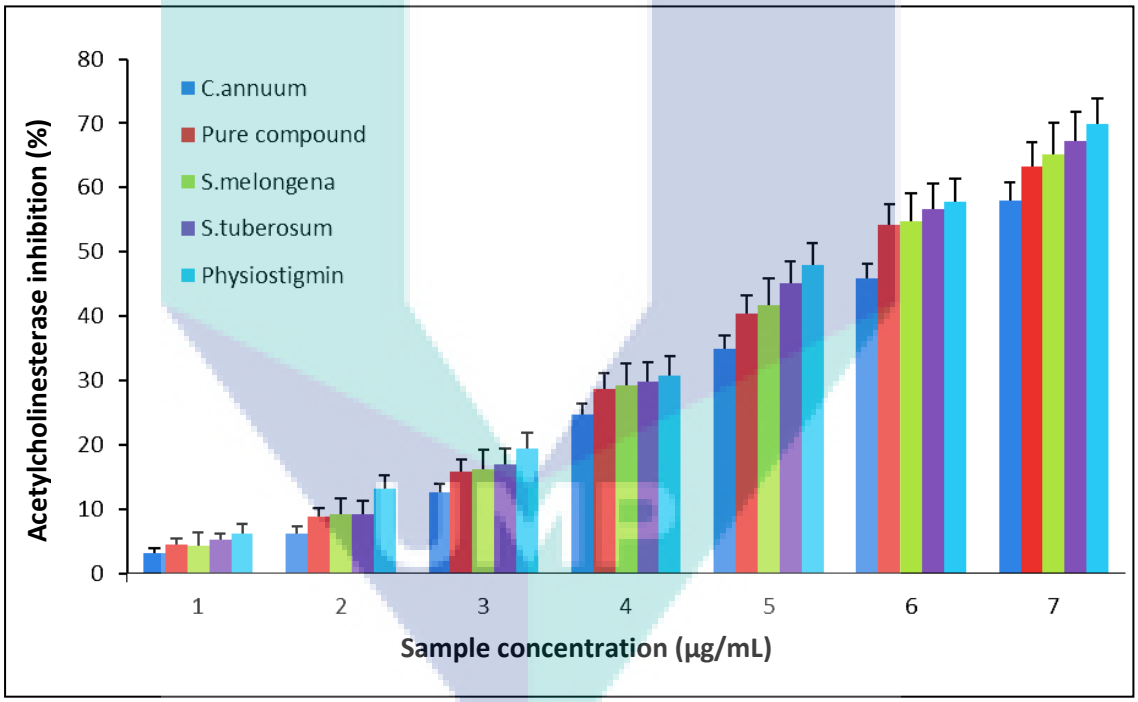


Figure 4.4: Acetylcholinesterase inhibitory activity of 3 plant extracts and the isolated compound using the physostigmine (Eserine) as standard.

4.8 ANTIACETYLCHOLINESTERASE ACTIVITY (TLC)

Acetylcholinesterase inhibitory activities of the fractions and isolated compound from three selected solanum species were evaluated by TLC bioautographic assay

(Zhongduo et al., 2009) and the positive results are shown in Figure 4.5. White spots on a purple background showed AChE inhibitory activities. It was found that the fraction from *S. tuberosum S. melongena* and *C. annuum*. Showed AChE inhibitory activities. Galantamine was used as a positive control which showed a clear white spot on a purple background. On the one hand, the results from TLC bioautographic assay further confirmed the anti-AChE activities of the fractions from all the three solanum species. On the other hand, it also provided us further detailed informations about the number of white spots (except for false-positive spots) on a purple background implied that there may be the same number of AChE inhibitors in plant extracts. After comparing with the false-positive assay, it was seen that the white spots on a purple background were seen only in the TLC bioautographic assay, and not in the false positive assay. This meant that the false-positive results caused by inhibition of 1-naphthol reaction with Fast Blue B salt barely existed, and the results of TLC assay were reliable.

Zhongduo et al. (2012) studied the anti-AChE activity for 31 Chines herbal plants alkaloidal extracts using TLC bioautographic method. Among 31 plants from different famlies. Only some of them inhibited the actylcholinesterase by exhibitng the white spot on the purple background of the TLC plate. Though the Zhongduo et al. (2012) used different ration of eluted solvent (chloroform–methanol 15:1 v/v) but still the separation of the compounds on TLC in the form of white spots are clear and in similar pattern.

S. tuberosum and *S. melongena* fraction inhibits more as compare to *C. annuum*. Galantamine was used as a positive control because it is a well known inhibitor of AChE (Ezoulin et al., 2006). The sample and rapid thin-layer chromatography-based assays were developed by Zhongduo et al. (2009) for different plant samples.

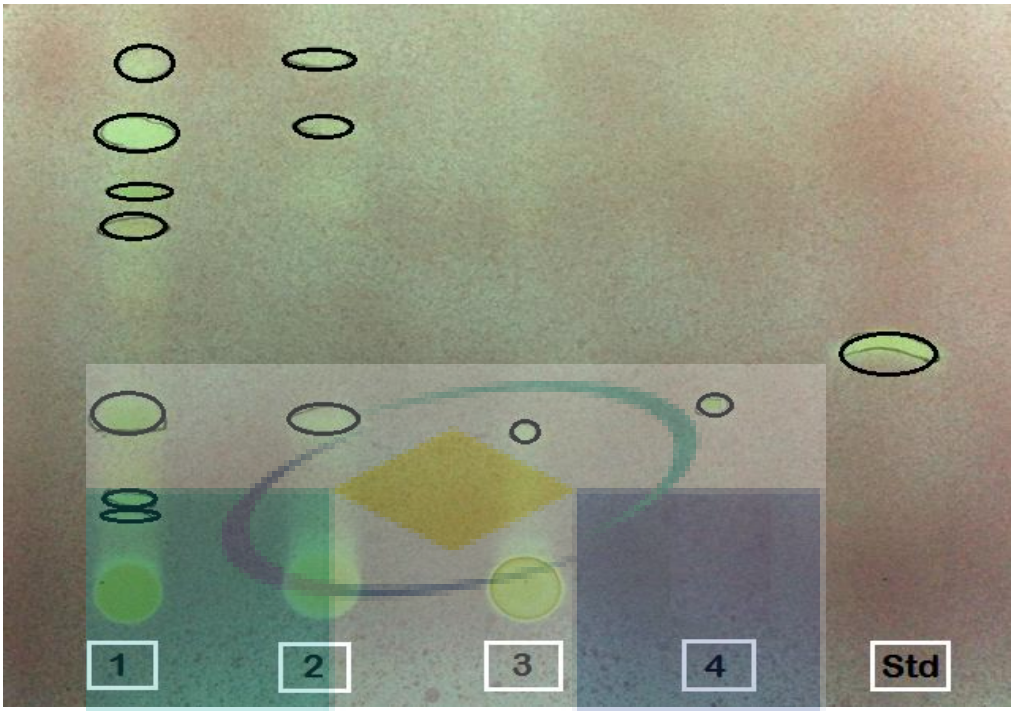


Figure: 4.5: TLC bioautograph showing the inhibition of AChE by isolated compound and 3 plant extracts. Spot 1 = *S. tuberosum*, 2 = *S. melongena*, 3 = *C. annuum* and 4 = Isolated compound, Galantamine was used as standard (Std). Plate was eluted by chloroform- methanol (6:4 v/v).

4.9 CORRELATION OF DPPH SCAVENGING ACTIVITY WITH ANTI-AChE

The correlation between DPPH radical scavenging activity and anti-AChE of solanum species is shown in Figure 4.6. From the results of the present study it is revealed that anti-AChE activity of the extracts and the pure compound is significantly correlated with their antioxidant capacity (R^2= 0.9774).

Cole et al. (2005) reported that the antioxidants are responsible to quench the free radicals, its excess cause a condition which is known as oxidative stress. The oxidative stress has been observed in the early stage AD patients. Oxidative stress in AD is because of many factors such as aging and amyloid plaques. Although it is clear that the free mediated attack on lipids, proteins, and nucleic acids results in oxidative stress, and these free radicals can easily be controlled by using the food rich in antioxidants. It is clear from the above results the free radicals has a close corelation with anti-AChE activity.

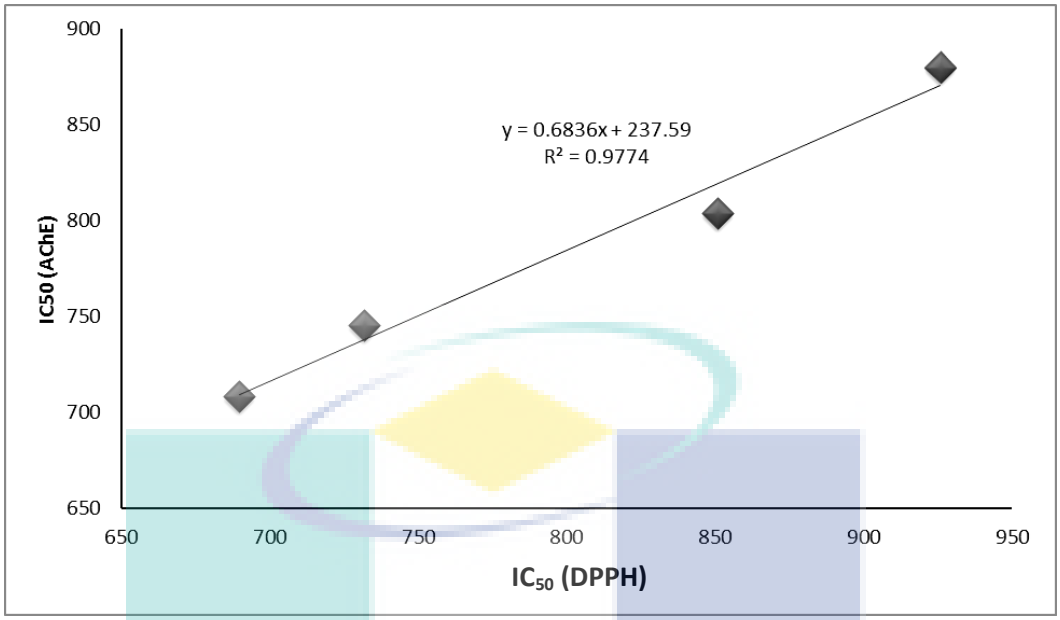


Figure 4.6: Correlation between anti-AChE and DPPH radical scavenging activity.

4.10 COMPOUND ISOLATION

Fractionation and chromatography of alkaloidal fraction D from non-edible parts of three solanum species, *S. tuberosum, S. melogena* and *C. annuum* resulted in the isolation of glycoalkaloidal compound from all the three samples in different ratios. Fraction D analyzed by HPLC in order to separate the alkaloidal compound, as shown in Figure 4.7.

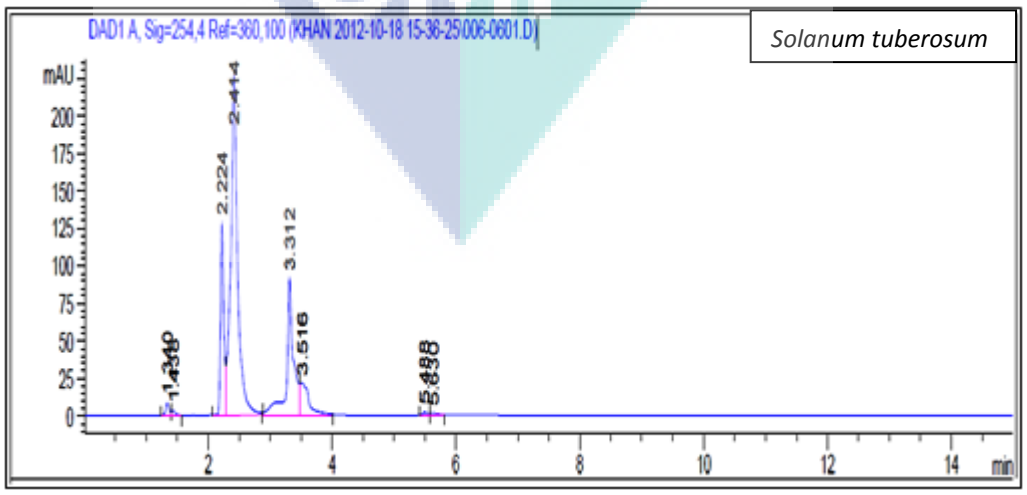
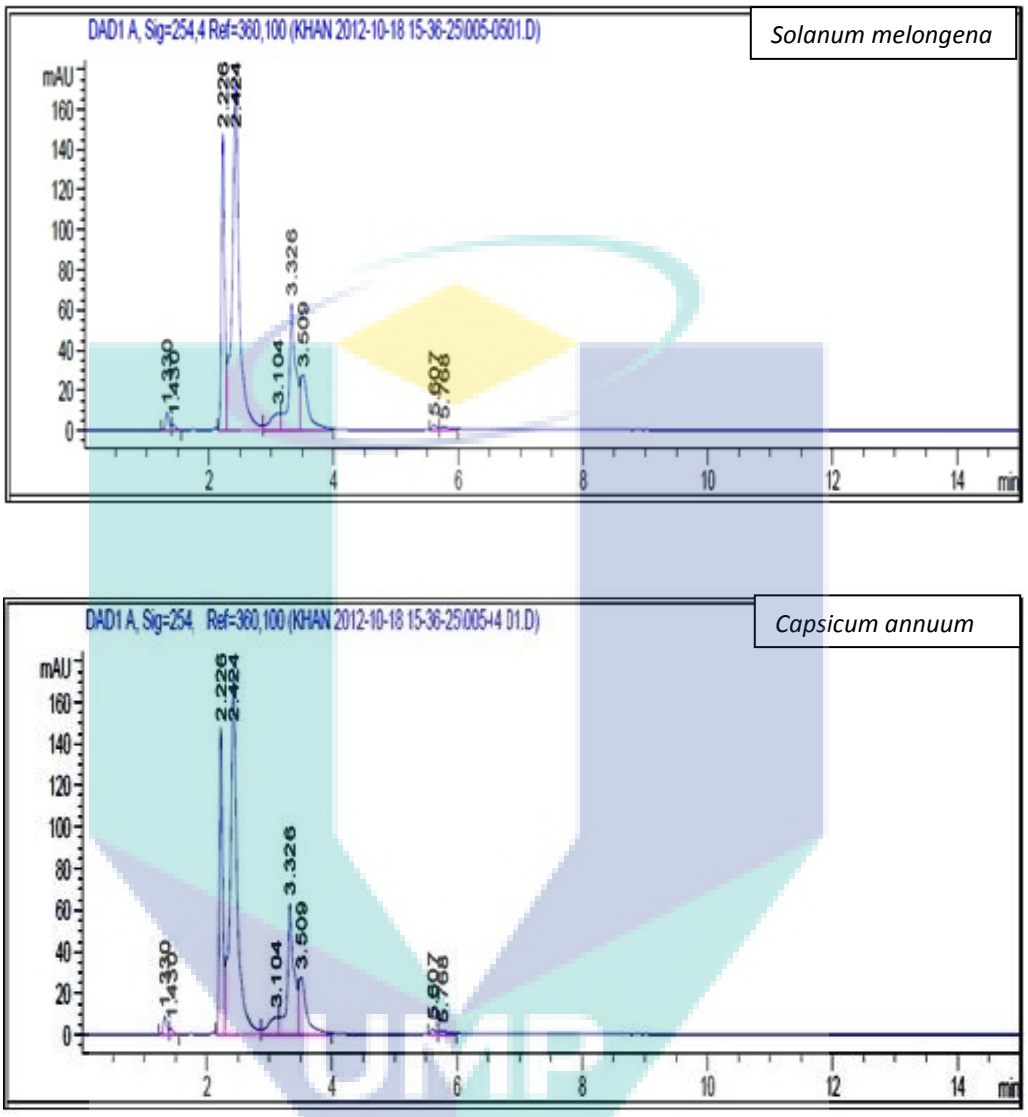


Figure: 4.7. HPLC chromatogram of alkaloidal compounds from non-edible parts of solanum species found in fraction D.

Figure: 4.7 Continued



Conditions: C18 reverse phase column with 250 mm × 4.6 mm, 20 µL injection volume, ACN: Tris-HCl(70:30) buffer as solvent. The most abundant peak eluting at 2.41and 2.42 were collected for further study.

The purity of the pooled fraction 2.41 and 2.42 from HPLC were confirmed TLC examination. The compound was subjected to LCMS and the molecular mass determined to be equal to that of α-solanine. Therefore, the fraction D was purified in mass scale by preparative HPLC to obtain sufficient amount of the pure compound for further study. The percent yield of the pure compound was calculated by the formula described in Appendix 1. The details are shown in Table 4.6.

Table 4.6: Isolation of pure compound α-solanine from non-edible parts of three solanum species by fractionation and HPLC.

Plant	Initial dry weight (g)	Weight after defatting (g)	Fraction D dry weight (g)	Pure compound (mg)	Pure compound % yield
S. tuberosum	500	489	4.68	3.80	7.6×10^{-4}
S. melongena	500	482	5.13	3.75	7.5×10^{-4}
C. annuum	500	485	3.97	3.50	7.0×10^{-4}

From the Table 4.6 it is evident that the recovery of the pure compound was almost similar in all 3 plant extracts. The recovery was the highest from *S. tubersum* followed by *S. melongena* and *C. annuum*. These results are also in agreement with Ieri et al. (2011), isolated the glycoalkaloids from potato using HPLC and MS. Wu et al. (2012) obtained 0.0049 (mg) % yield of the glycoalkaloid from potato peel using column chromatography. Neslihan (2006) isolated solanine and other glycoalkaloids from the *S. melongena* fruit using HLPC and GC-MS method, but non of them used non-edible parts of *S. melongena* to isolate alpha solanine. Andersson (1999) reported the isolation of alpha solanine from the green and red pepper by colorimetric method. This study reports the second time isolation of glycoalkaloid after Andersson (1999).

The purified compound was a white powder. The IR spectrum (Appendix 12, 13, and 14) displayed a broad absorption band in the region 3324-2820 cm^{-1} corresponding to the OH functional group. N-H stretching appeared at 1417 cm^{-1}, while stretching due to C-O was observed at 1022 cm^{-1}. Thus the IR data indicated the presence of OH, NH and CO groups in the compound.

The ^{1}H NMR spectrum (Appendix 16, and 17) of purified compound revealed no aromatic protons. A doublet with a coupling constant 5.25 Hz resonated at 5.39 was attributed to an olefinic proton at H-6 (Table 4.7). Another four signals of methyl protons at δ 0.99 (d, J=5.25), 1.07 (s, 3H), 0.91 (s, 6H) were assigned as H-18, H-19, H-21 and

H-27, respectively. The signals of methylene protons and methane were found at 1.25-2.23 (m, 26H).

Table: 4.7: ^{1}H NMR (500 MHz, MeOH) of the purified compound.

Position	δH (ppm) Int. Mult. J
3	2.23 (1H, m)
6	5.39 (1H, d, J=5.25)
18	0.99 (3H,d, J= 3.6)
19	1.07 (3H,s)
21, 27	0.91(6H,s)
26	2.48, 2.35 (2H, m)
9 ×CH_2, 8 ×CH	1.25 – 2.23 (26H, m)
1″	5.23 (1H, s)
1′	5.51 (1H, s)
2 ×CH_2, 13 ×CH	3.43 - 4.62 (17H, m)
6′	1.25 (3H, s)

Although spectral data of many alkaloids have been published, the literature survey shows that the complete ^{1}H NMR assignment of the glycosidic part is unavaliable (Puri et al., 1994). So the ^{1}H spectrum of sample was compared with the standard (*α*-solanine from Sigma-Aldrich), and both the spectra showed almost similair pattern (Appendix 15), which support to assign it as *α*-solanine, though ^{13}C NMR data is needed for further confirmation. Unfrotunately, the amount of purified sample required for ^{13}C NMR analysis was not obtained. Therefore, ^{13}C NMR data for the purified compound was notpresented.

The structure of this compound was further characterized by LC-MS analysis. The TOF MS/MS spectrum of purified *α*-solanine (Figure 4.8) was generated by fragmentation of its $[M + H]^{+}$ ions using relative collision energy (RCE) values of 60V (Figure 4.9).

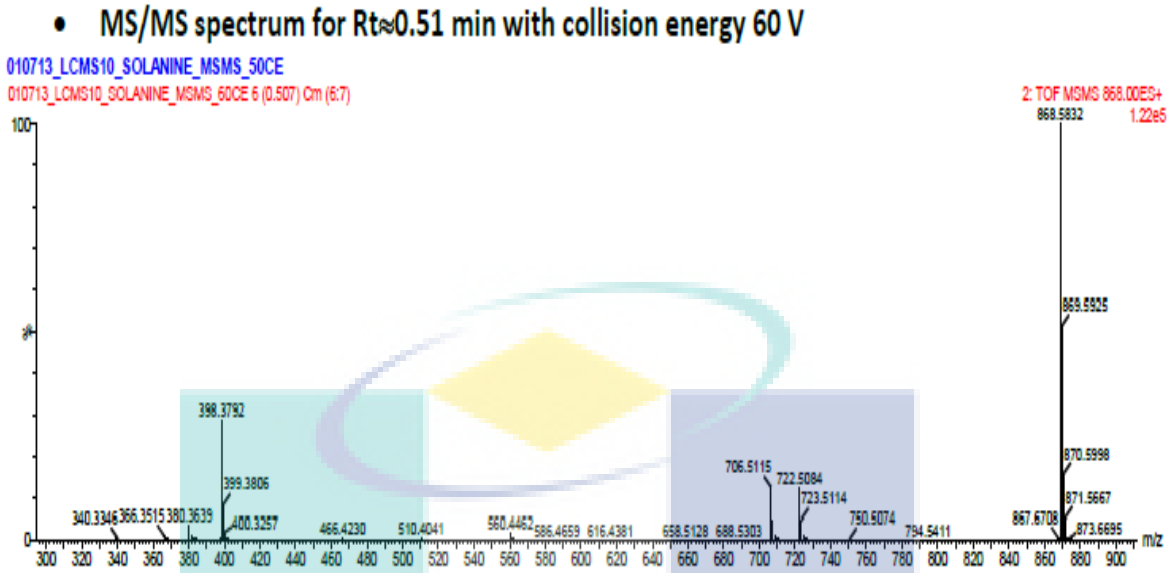
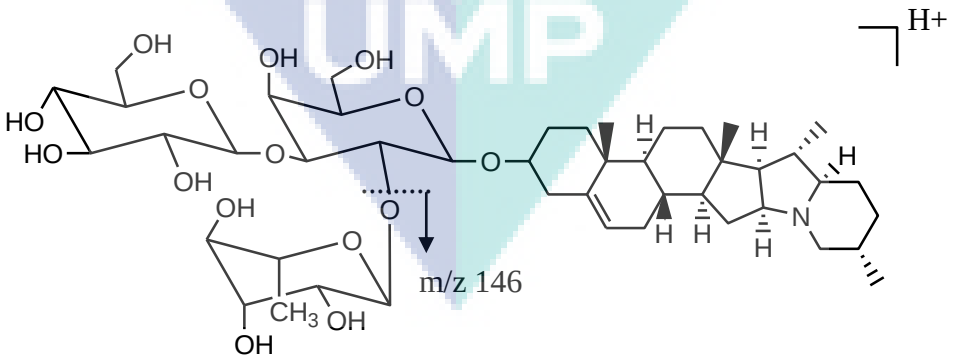


Figure 4.8: Mass fragmentation spectrum of the isolated compound from non-edible parts analyzed by TOF MS/MS with ACQ-PDA detector.

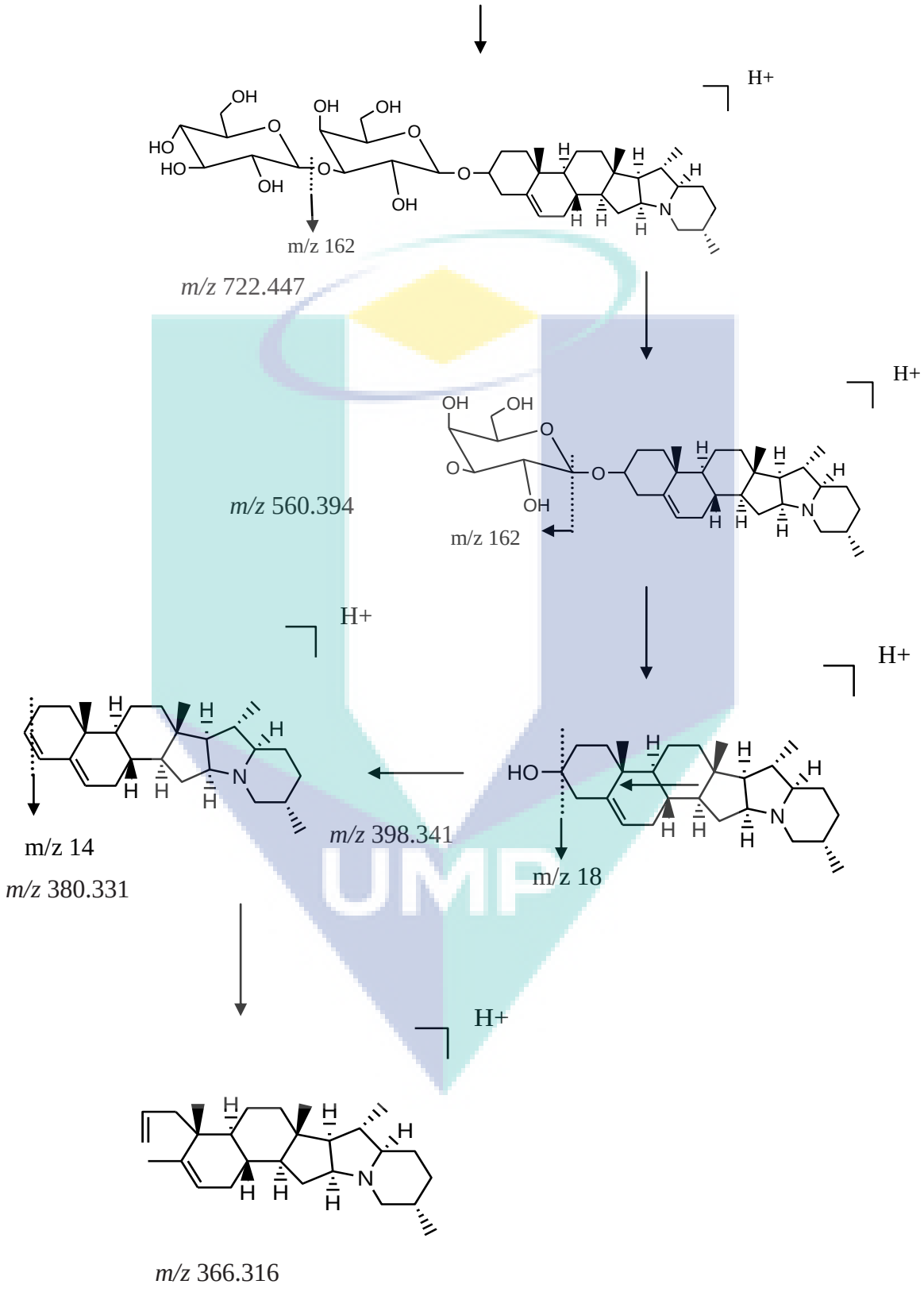
The use of an optimized RCE allowed a portion of precursor ions to be observed in the high energy collision-induced dissociation (HCD) spectrum. However, since ions generated using the HCD were analyzed in the ion trap Fourier transform mass spectrometry (FTMS), high mass accuracy data was acquired for product ions.



m/z 868.5057

Figure 4.9: Mass fragmentation pathway for α-solanine. The accurate mass was determined by TOF MS/MS with ACQ-PDA detector.

Figure 4.9: Continued



The product ion at m/z 722 is due to a facile loss of the monosaccharide, L-rhamnose and similarly, the ion at *m*/*z* 706 is attributed to a loss of the D-glucose monosaccharide. The MS^3 spectra were obtained using CID by trapping and fragmenting of the ions, *m*/*z* 722. The product ion, *m*/*z* 560, from fragmentation of the ion, *m*/*z* 722, is attributed to a loss of the D-glucose moiety. The next stage in the fragmentation pathways for toxins is the loss of the last monosacharride from the ion, *m*/*z* 560, to produce the aglycon ion, *m*/*z* 398, which was the base peak in the MS^4 spectra, together with the water-loss ion, *m*/*z* 380, and an ion at *m*/*z* 366, formed by the loss of methanol from the A-ring, complying with Michael et al. (2010).

Generally, fragmentation of the six-ring steroidal backbone produced low abundant ions other than the ions at m/z 98 and 126, which are due to fragmentation of the E-ring. The proposed pathway for mass fragmentation of α-solanine is shown in Figure 4.10, with the accurate mass values that obtained using HCD MS/MS in the Orbitrap. The quality of the accurate mass determinations using the Orbitrap MS is demonstrated by the fact that all the precursor and product ions greater than m/z 300 were determined with error values less than 1 ppm. Mass spectra and elemental analysis of purified α-solanine for all the three plants, are shown in appendices 19, 20, and 21.

The molecular ion peak at m/z 868 corresponding to a molecular formula $C_{45}H_{73}NO_{15}$. On the bases of its physical properties and spectroscopic data compound was assigned as α-solanine. This compound had been isolated from peels, sprouts, leaves, tuber and roots of *S. tuberosum* by Friedman (2006) and Dinan et al., (2001). While in *S. melongena*, it has been isolated from the eggplant tuber by Chen and Miller (2001) but first time isolated from the non-edible parts of eggplant. In case of *C. annum*, limited literature is available that discuss about the glycoalkaloids in capsicum (Andersson et al., 2003), while isolation of α-solanine from *C. annuum* is not reported. So the attempt to isolate the α-solanine from the non-edible parts of the *C. annuum* succeeded although the % yield is low.

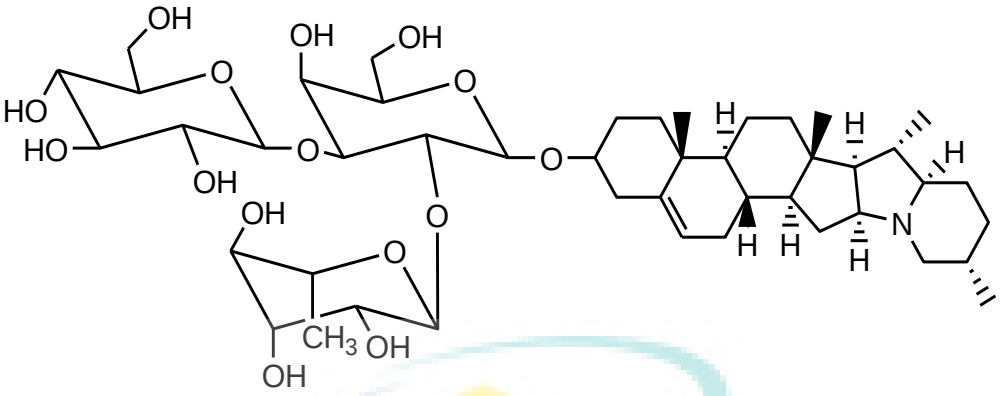


Figure 4.10: Chemical structure of α-solanine.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

This chapter summarizes and consolidates the detailed discussion and conclusion that have already been presented. In this study, a total of 32 plants were screened for bioactive compounds and out of them 3 plants with highest active constituents were chosen for further study. The non-edible parts of these 3 plants were extracted and subsequently fractioned to get the alkaloidal compound. The crude and the pure compound were subjected to determination of TPC, TFC, anti-AchE and antioxidant activities. All the crudes and the pure compound demonstrated almost similar values for the above determinations.

The anti-AchE activity demonstrated by both crude and the purified compound indicated that the most active compound belongs to alkaloids. Different literature showed that quaternary nitrogen is necessary for the strong AChE inhibitory activity by alkaloids. Spectroscopic studies of the purified compound indicated the compound to be α-solanine, an alkaloid that contains quarternary nitrogen in its structure. Although the purified compound is already known, the isolation of this compound from the nonedible part of these plants is done in this study for the first time. Thus the study demonstrated that the non-edible part of these 3 plants can play a vital role in maintaining good health specially by providing anti-ageing and memory protective suplements.

5.2 RECOMMENDATIONS FOR THE FUTURE RESEARCH

There is a need to broaden the research on alkaloids, particularly in solanum species in order to get potential candidates for curing different disorders. Although there are many reports published on these plants but many of these compounds are yet to be investigated for their nutraceutical and pharmacological activities. Structure-activity relationship (SAR) plays a vital role in understanding the molecular mechanism of biochemical activities. It can suggest the possible biological activity of the compounds.

Oxidative stress considers to be hazardous to the health, as it is the initial point for many neurodegenerative diseases. In order to prevent the population from fatal diseases as dementia and oxidative stress, there is a dire need to explore clear relation between them and set their precautions.

Any natural product or isolated compound that can effectively inhibit Human AChE and BuChE can be a drug candidate for the treatment of AD. Therefore, further research on the search for potent inhibitor of AChE and BuChE may result in obtaining more reliable drug candidates to control memory loss with greater success.

The potency of inhibitor depends on the affinity of binding of the compound on the active site. A compound with high binding affinity (low K_m value) can occupy all the active sites and can exhibit greater effect. Further research is needed to explore inhitors with high binding affinity. Kinetic study of the isolated compound is also required to know the binding affinity of the compounds.

Before selecting a compound as a drug for human, an animal study is warrented followed by human trials and pharmacokinetic study. Therefore, it is recommended that the 3 screened plants can be used for futher elaborate research to formulate an effective drug for the treatment of Alzheimer's disease.

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APPENDIX 1

CALCULATION OF PERCENT YIELD

The percent yield of isolated compound is calculated using the following formula.

Yield (%) = $W_{\square}$ / Wi x 100

Where

$W_{\square}$ = Weight of isolated compund(Solanine) obtained

Wi = Total weight of fraction D containing alkaloids

APPENDIX 2

STATISTICAL ANALYSIS OF DPPH RADICAL SCAVENGING ACTIVITY (%) INHIBITION AND IC_{50}

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DPPH % inhibition	Between Groups	474.230	4	118.557	1044.068	.000
	Within Groups	1.136	10	.114		
	Total	475.365	14			
DPPH IC_{50}	Between Groups	61613.079	4	15403.270	33824.595	.000
	Within Groups	4.554	10	.455		
	Total	61617.633	14			

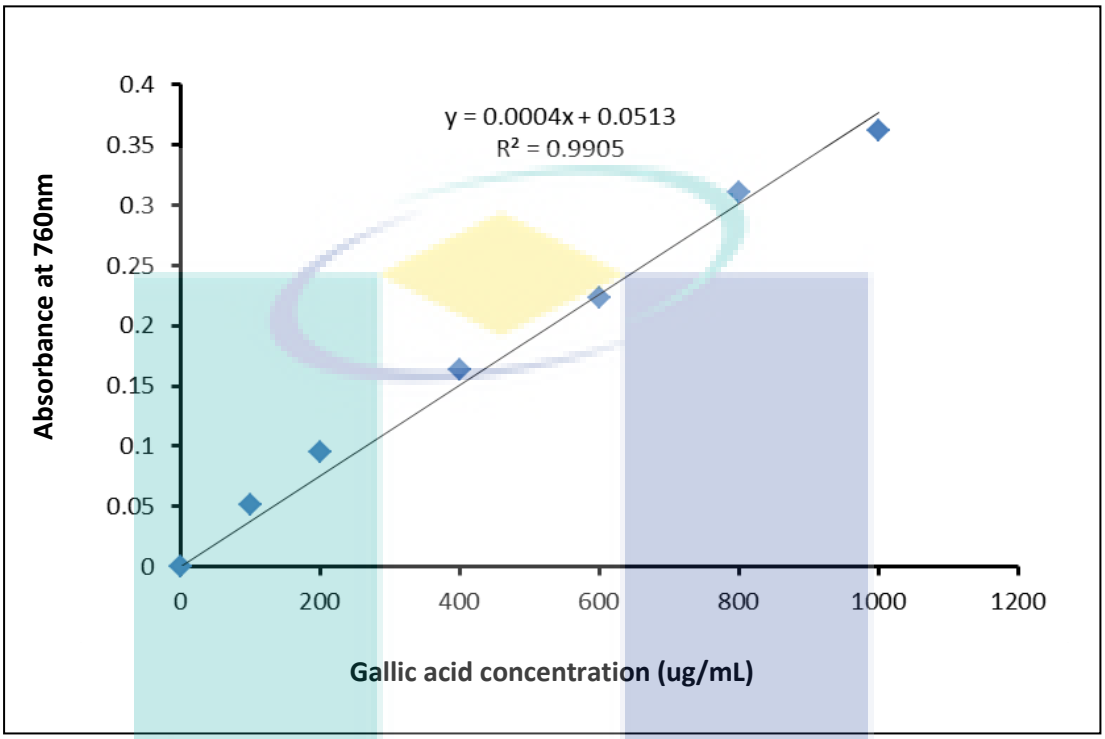
APPENDIX 3

STATISTICAL ANALYSIS OF TOTAL PHENOLIC CONTENTS (TPC) AND TOTAL FLAVONOID COMPOUNDS (TFC)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TPC	Between Solanum species	10842.297	3	3614.099	2780076.058	.000
	Within Solanum species	.010	8	.001		
	Total	10842.307	11			
TFC	Between Solanum species	8670.169	3	2890.056	1156022.520	.000
	Within Solanum species	.020	8	.002		
	Total	8670.189	11			

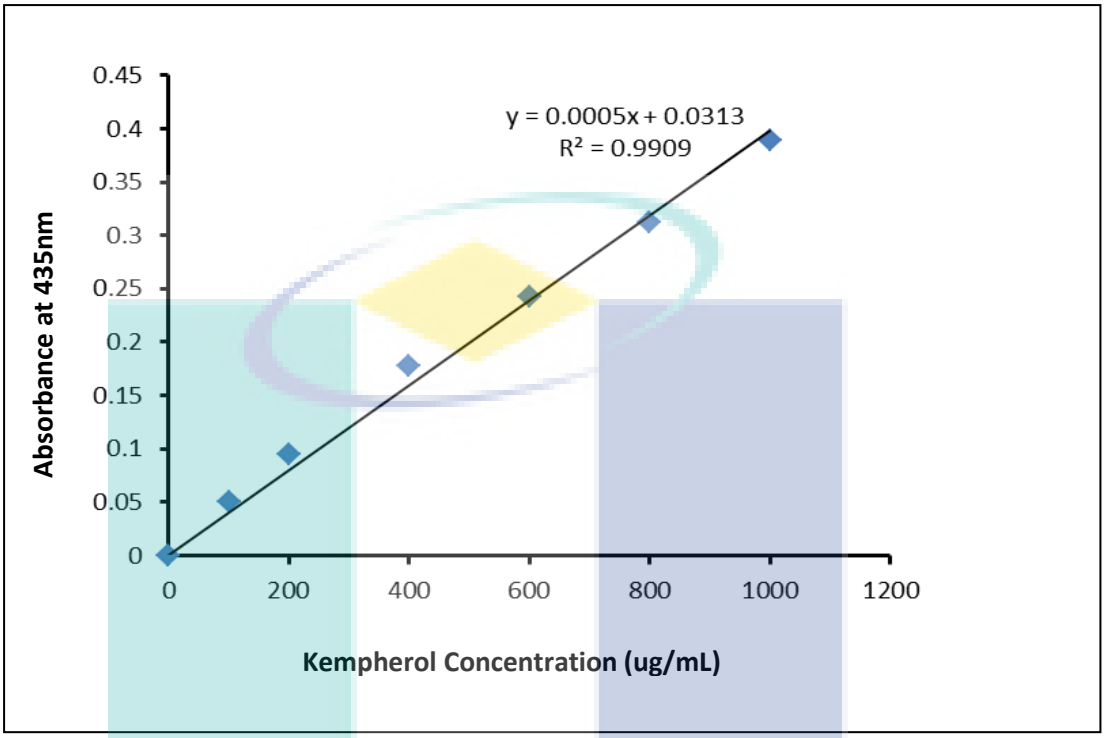
APPENDIX 4



(values in mean ± SD, n= 3)

Figure 1: Calibration curve of Gallic acid for the calculation of TPC

APPENDIX 5



(values in mean ± SD, n= 3)

Figure 2: Calibration curve of Kaempherol for the calculation of TFC

APPENDIX 6

STATISTICAL ANALYSIS OF CORRELATION OF ANTIOXIDANT ACTIVITY (DPPH SCAVENGING ACTIVITY) WITH THE INHIBITION OF ACETYLCHOLINESTERASE ACTIVITY

Correlations

		AChE IC_{50}	DPPH IC_{50}
AChE EIC_{50}	Pearson Correlation	1	.973**
	Sig. (2-tailed)		.000
	N	12	12
DPPH IC_{50}	Pearson Correlation	.973**	1
	Sig. (2-tailed)	.000	
	N	12	12

**. Correlation is significant at the 0.05 level (2-tailed).

APPENDIX 7

STATISTICAL ANALYSIS OF CORRELATION OF AChE WITH TPC, AND TFC

Correlations

		AChE IC_{50}	TPC
AChE IC_{50}	Pearson Correlation	1	-.756**
	Sig. (2-tailed)		.004
	N	12	12
TPC	Pearson Correlation	-.756**	1
	Sig. (2-tailed)	.004	
	N	12	12

**. Correlation is significant at the 0.05 level (2-tailed).

Correlations

		AChEIC_{50}	TFC
AChEIC_{50}	Pearson Correlation	1	-.881**
	Sig. (2-tailed)		.000
	N	12	12
TFC	Pearson Correlation	-.881**	1
	Sig. (2-tailed)	.000	
	N	12	12

**. Correlation is significant at the 0.05 level (2-tailed).

APPENDIX 8

STATISTICAL ANALYSIS OF CORRELATION OF DPPH SCAVENGING ACTIVITY WITH TPC, AND TFC,

Correlations

		$DPPHIC_{50}$	TPC
$DPPHIC_{50}$	Pearson Correlation	1	-.897**
	Sig. (2-tailed)		.000
	N	12	12
TPC	Pearson Correlation	-.897**	1
	Sig. (2-tailed)	.000	
	N	12	12

**. Correlation is significant at the 0.05 level (2-tailed).

Correlations

		DPPH IC_{50}	TFC
DPPH IC_{50}	Pearson Correlation	1	-.973**
	Sig. (2-tailed)		.000
	N	12	12
TFC	Pearson Correlation	-.973**	1
	Sig. (2-tailed)	.000	
	N	12	12

**. Correlation is significant at the 0.05 level (2-tailed).

APPENDIX 9

STATISTICAL ANALYSIS OF CORRELATION OF TOTAL PHENOLIC WITH TOTAL FLAVONOID CONTENT

Correlations

		TPC	TFC
TPC	Pearson Correlation	1	.898**
	Sig. (2-tailed)		.000
	N	12	12
TFC	Pearson Correlation	.898**	1
	Sig. (2-tailed)	.000	
	N	12	12

**. Correlation is significant at the 0.05 level (2-tailed).

APPENDIX 10

STATISTICAL ANALYSIS OF DPPH RADICAL SCAVENGING ACTIVITY (%) INHIBITION AND IC_{50}

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DPPH % inhibition	Between Groups	474.230	4	118.557	1044.068	.000
	Within Groups	1.136	10	.114		
	Total	475.365	14			
DPPH IC_{50}	Between Groups	61613.079	4	15403.270	33824.595	.000
	Within Groups	4.554	10	.455		
	Total	61617.633	14			

APPENDIX 11

STATISTICAL ANALYSIS OF ANTI-ACETYLCHOLINESTERASE (% INHIBITION) OF STUDIED PLANTS

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
AChE % inhibition	Between Plant species	9520.549	41	232.209	345.137	.043
	Within Plant species	.673	1	.673		
	Total	9521.221	42			

APPENDIX 12

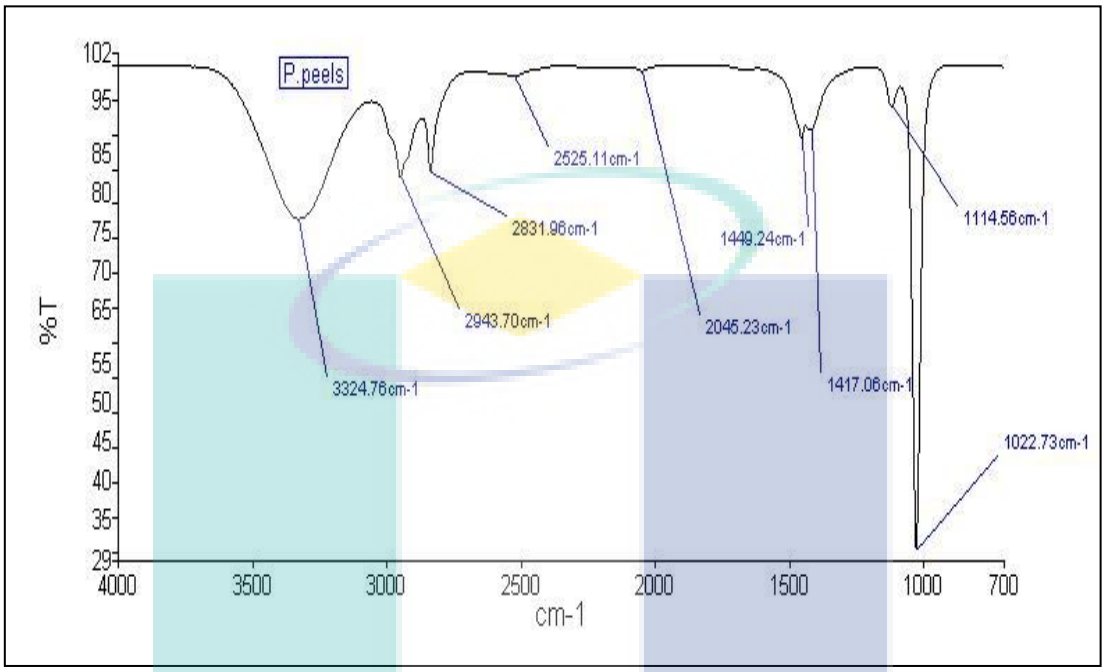


Figure 3: FTIR spectrum of α-solanine from *s.tuberosum* non-edible part

APPENDIX 13

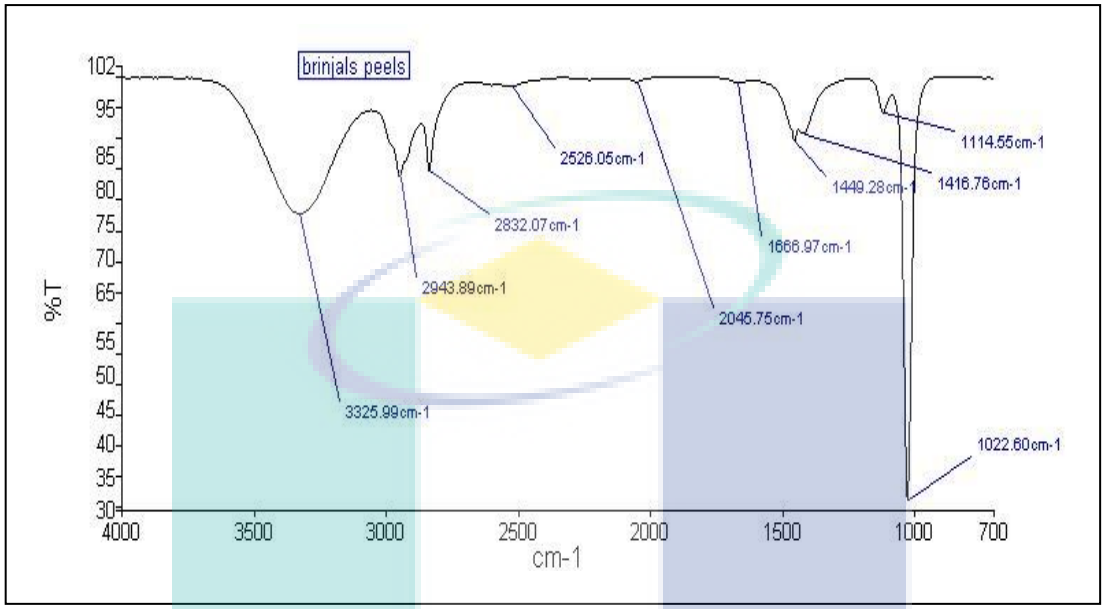


Figure 4: FTIR spectrum of α-solanine from *s.melongena* non-edible part

APPENDIX 14

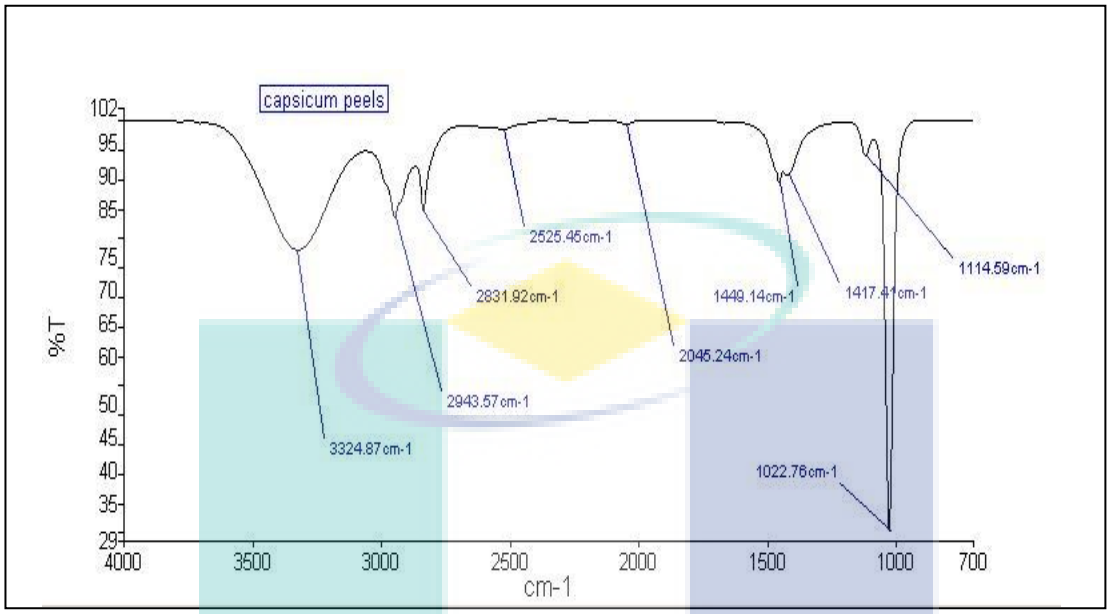


Figure 5: FTIR spectrum of α-solanine from *c.annuum* non-edible part

APPENDIX 15

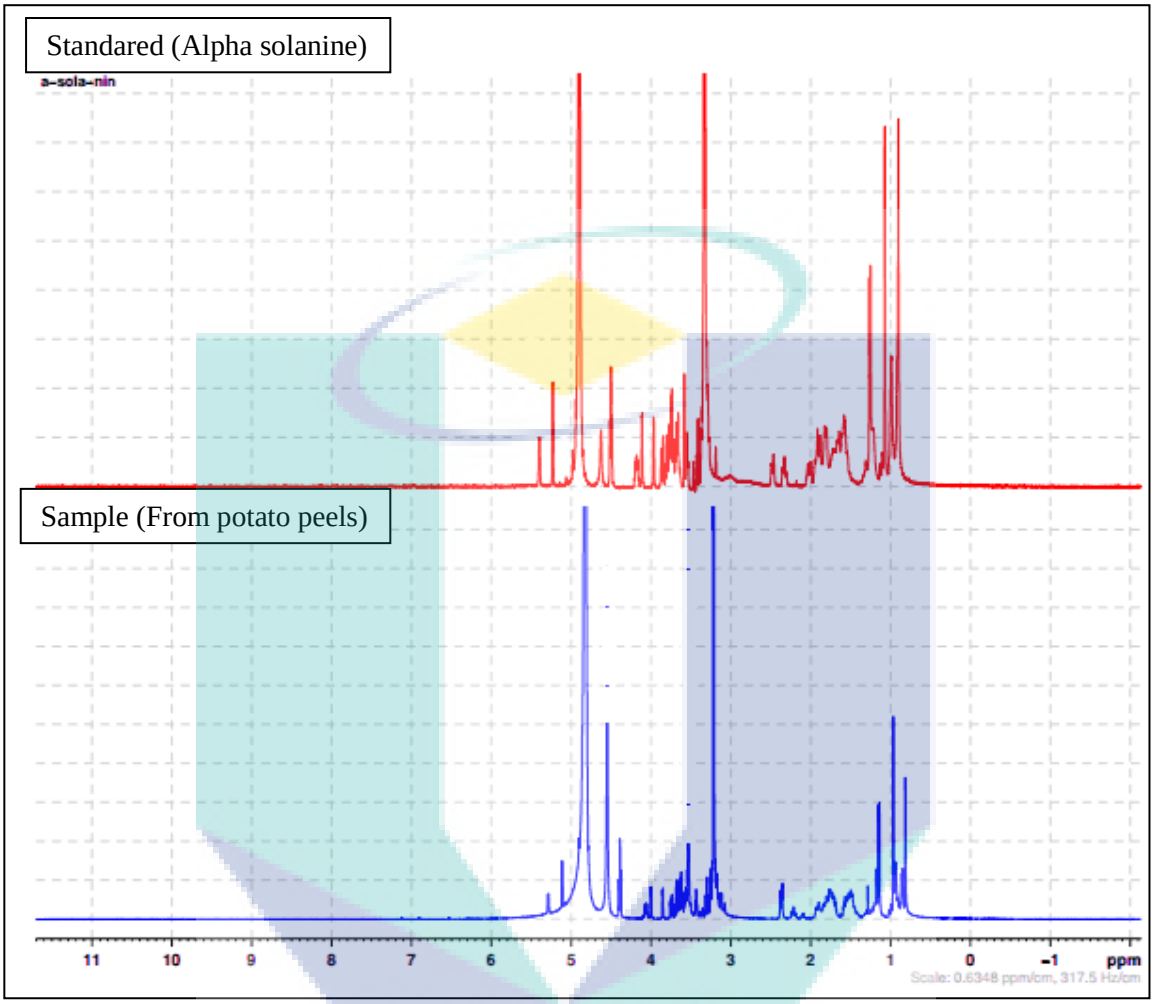


Figure 6: Comparison of ^{1}H NMR spectrum of α-solanine (standard) with sample of *S.tuberosum* peels

APPENDIX 16

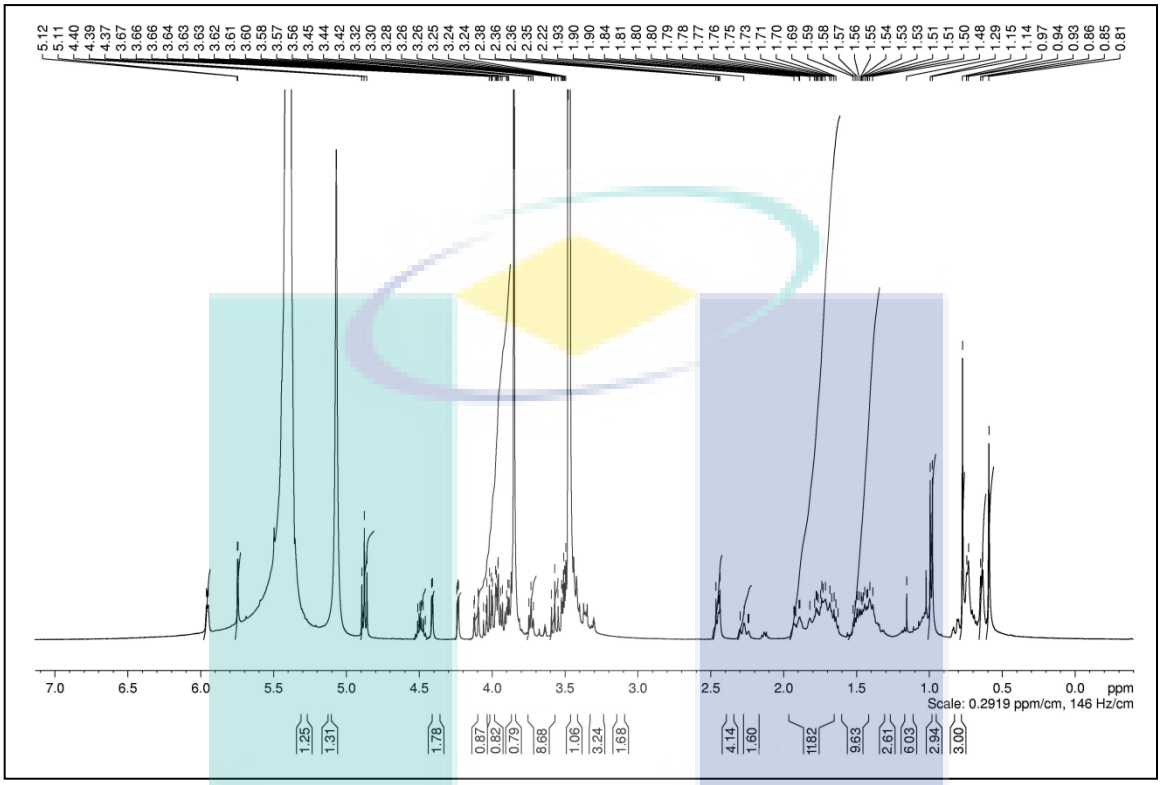


Figure 7: ^{1}H NMR spectrum of α-solanine from *S. melongena* non-edible parts

APPENDIX 17

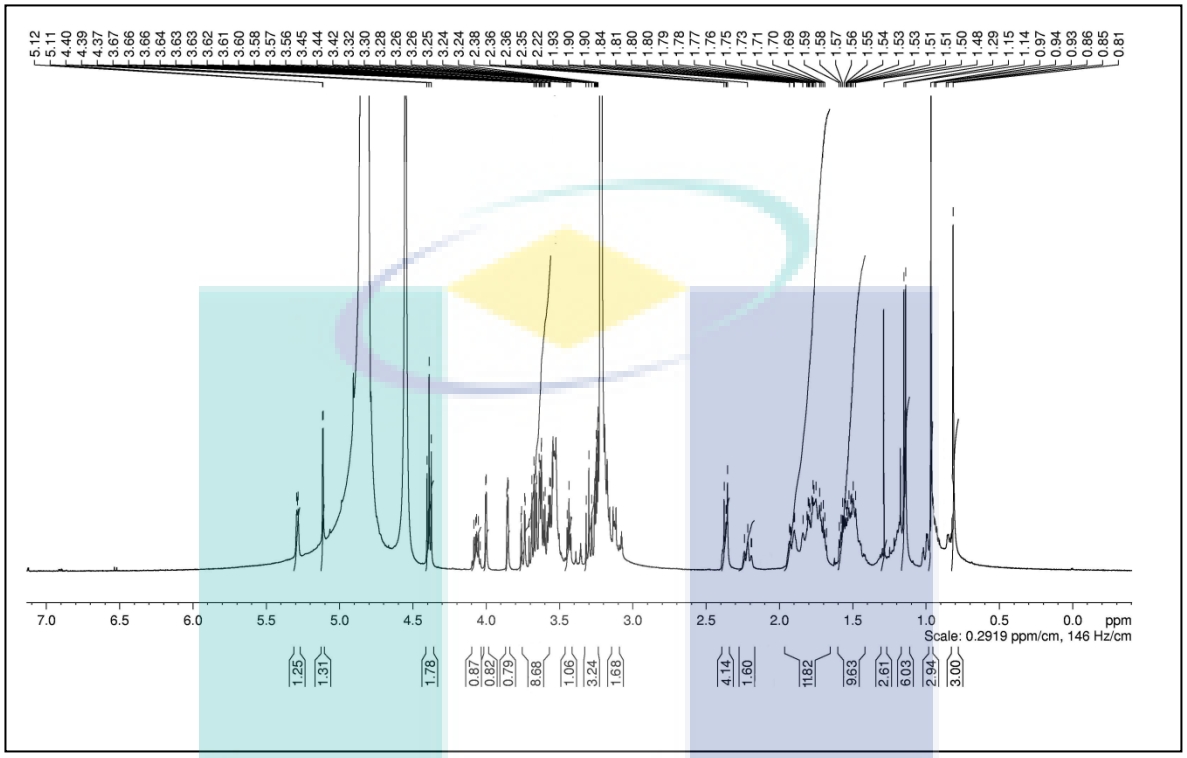


Figure 8: ^{1}H NMR spectrum of α-solanine from *C. annuum* non-edible parts

APPENDIX 18

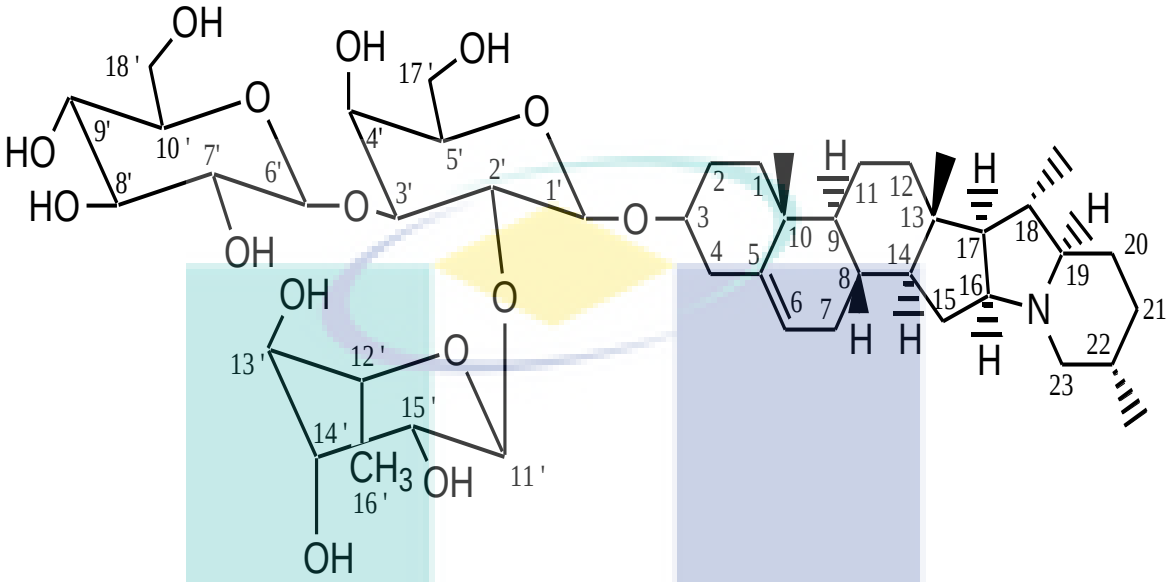


Figure 9: Chemical structure of purified α-solanine showing the numbering order used to assign the strucutre from ^{1}H NMR spectrum.

APPENDIX 19

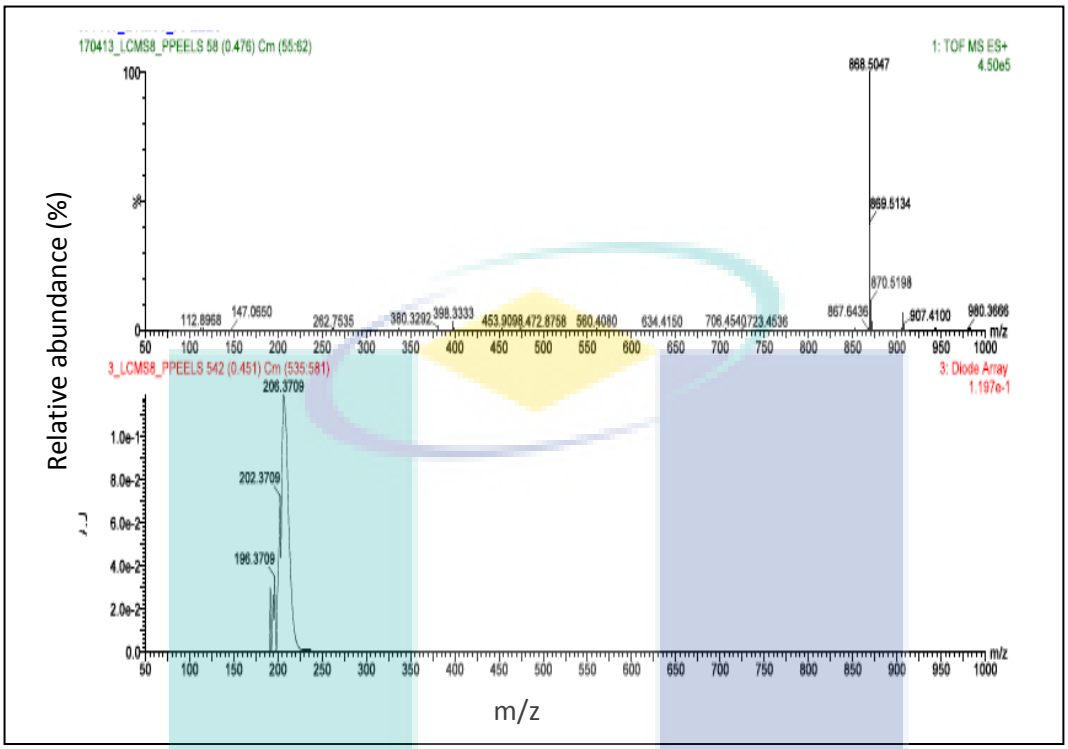


Figure 9: Mass spectrum of *s.tuberosum* peels

ELEMENTAL COMPOSITION FOR 868.5048 m/z FROM *S. tuberosum* PEELS

Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

3461 formula(e) evaluated with 10 results within limits (up to 50 closest results for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
868.5047	868.5090	-4.3	-5.0	1.5	C34 H74 N7 O18	27.3	0.011	99.88	34	74	7	18
	868.5018	2.9	3.3	5.5	C40 H74 N3 O17	32.3	5.035	0.65	40	74	3	17
	868.5032	1.5	1.7	10.5	C41 H70 N7 O13	33.2	5.931	0.27	41	70	7	13
	868.5058	-1.1	-1.3	9.5	C45 H74 N O15	34.2	6.904	0.10	45	74	1	15
	868.5072	-2.5	-2.9	14.5	C46 H70 N5 O11	34.8	7.508	0.05	46	70	5	11
	868.5085	-3.8	-4.4	19.5	C47 H66 N9 O7	35.4	8.090	0.03	47	66	9	7
	868.5013	3.4	3.9	23.5	C53 H66 N3 O6	37.1	9.866	0.01	53	66	3	6
	868.5026	2.1	2.4	28.5	C54 H62 N9 O2	37.4	10.094	0.00	54	62	9	2
	868.5053	-0.6	-0.7	27.5	C58 H66 N3 O4	37.3	10.077	0.00	58	66	3	4
	868.5067	-2.0	-2.3	32.5	C59 H62 N7	37.6	10.340	0.00	59	62	7	

APPENDIX 20

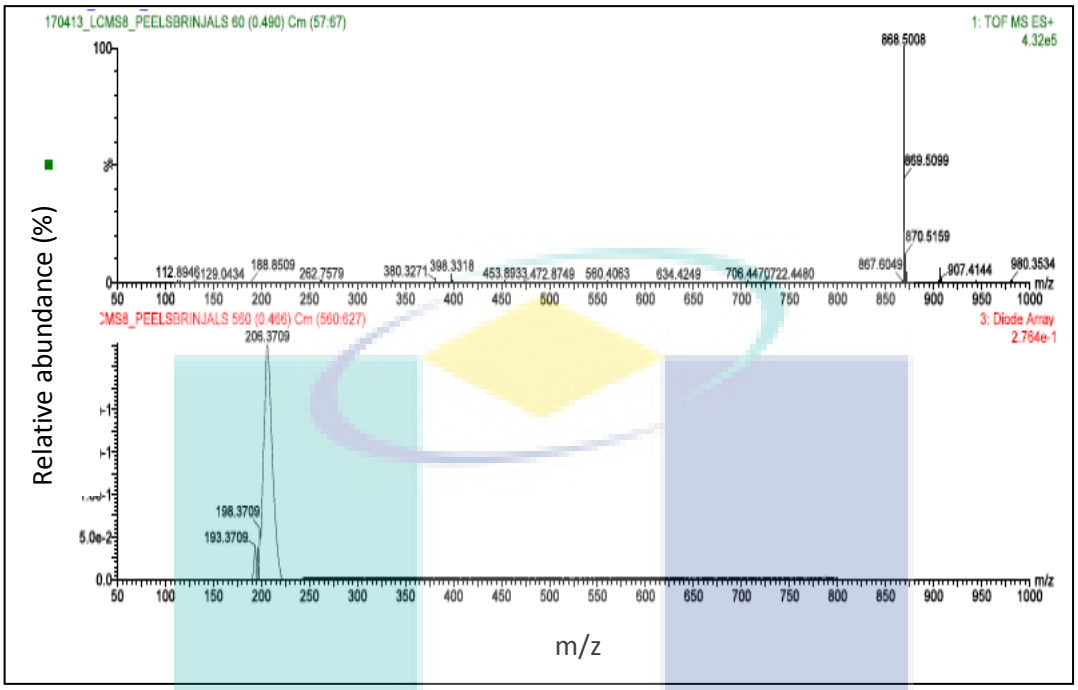


Figure 10. Mass spectrum of non-edible part of *S. Melongena*

ELEMENTAL COMPOSITION FOR 868.5048 m/z OF NON-EDIBLE PART OF *S.melongena*

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
3459 formula(e) evaluated with 19 results within limits (up to 50 closest results for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
868.5008	868.5090	-8.2	-9.4	1.5	C34 H74 N7 O18	40.7	5.036	0.65	34	74	7	18
	868.4978	3.0	3.5	1.5	C35 H74 N5 O19	40.3	4.704	0.91	35	74	5	19
	868.4991	1.7	2.0	6.5	C36 H70 N9 O15	35.7	0.033	96.71	36	70	9	15
	868.5018	-1.0	-1.2	5.5	C40 H74 N3 O17	40.0	4.340	1.30	40	74	3	17
	868.5032	-2.4	-2.8	10.5	C41 H70 N7 O13	41.4	5.809	0.30	41	70	7	13
	868.4933	7.5	8.6	13.5	C43 H66 N9 O10	43.6	7.972	0.03	43	66	9	10
	868.5058	-5.0	-5.8	9.5	C45 H74 N O15	43.4	7.722	0.04	45	74	1	15
	868.5072	-6.4	-7.4	14.5	C46 H70 N5 O11	44.2	8.593	0.02	46	70	5	11
	868.5085	-7.7	-8.9	19.5	C47 H66 N9 O7	45.0	9.384	0.01	47	66	9	7
	868.4959	4.9	5.6	14.5	C47 H70 N3 O12	44.7	9.092	0.01	47	70	3	12
	868.4973	3.5	4.0	19.5	C48 H66 N7 O8	45.4	9.736	0.01	48	66	7	8
	868.5000	0.8	0.9	18.5	C52 H70 N O10	46.0	10.317	0.00	52	70	1	10
	868.5013	-0.5	-0.6	23.5	C53 H66 N5 O6	46.4	10.765	0.00	53	66	5	6
	868.5026	-1.8	-2.1	28.5	C54 H62 N9 O2	46.8	11.213	0.00	54	62	9	2
	868.5053	-4.5	-5.2	27.5	C58 H66 N3 O4	47.2	11.554	0.00	58	66	3	4
	868.5067	-5.9	-6.8	32.5	C59 H62 N7	47.6	11.962	0.00	59	62	7	
	868.4941	6.7	7.7	27.5	C59 H66 N O5	47.7	12.048	0.00	59	66	1	5
	868.4954	5.4	6.2	32.5	C60 H62 N5 O	48.0	12.324	0.00	60	62	5	1
	868.5094	-8.6	-9.9	31.5	C63 H66 N O2	47.9	12.270	0.00	63	66	1	2

APPENDIX 21

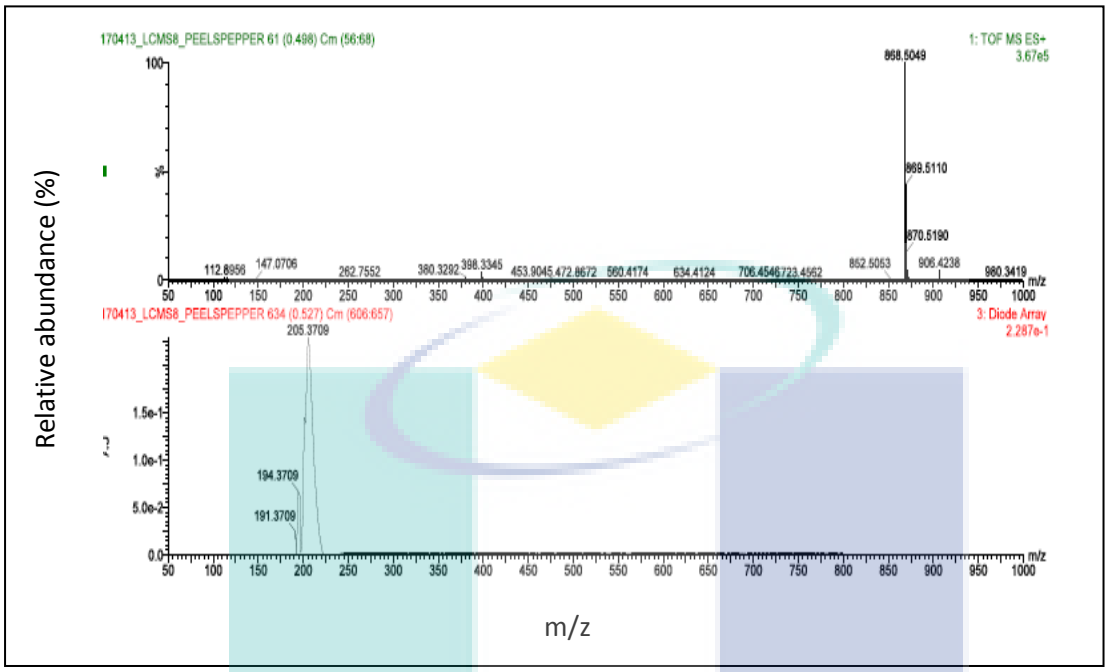


Figure 11: Mass spectrum of non-edible part of *S. annuum*

ELEMENTAL COMPOSITION FOR 868.5048 m/z OF NON-EDIBLE PART OF *C. annuum*

Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

3461 formula(e) evaluated with 10 results within limits (up to 50 closest results for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
868.5049	868.5090	-4.1	-4.7	1.5	C34 H74 N7 O18	29.5	2.701	6.71	34	74	7	18
	868.5018	3.1	3.6	5.5	C40 H74 N3 O17	26.9	0.144	86.63	40	74	3	17
	868.5032	1.7	2.0	10.5	C41 H70 N7 O13	29.7	2.970	5.13	41	70	7	13
	868.5058	0.9	1.0	9.5	C45 H74 N O15	31.4	4.643	0.96	45	74	1	15
	868.5072	-2.3	-2.6	14.5	C46 H70 N5 O11	32.4	5.640	0.36	46	70	5	11
	868.5085	-3.6	-4.1	19.5	C47 H66 N9 O7	33.3	6.496	0.15	47	66	9	7
	868.5013	3.6	4.1	23.5	C53 H66 N5 O6	35.3	8.534	0.02	53	66	5	6
	868.5026	2.3	2.6	28.5	C54 H62 N9 O2	35.6	8.827	0.01	54	62	9	2
	868.5053	-0.4	-0.5	27.5	C58 H66 N3 O4	35.6	8.825	0.01	58	66	3	4
	868.5067	-1.8	-2.1	32.5	C59 H62 N7	35.9	9.168	0.01	59	62	7	

APPENDIX 22

LIST OF PUBLICATIONS

Published Paper:

Azhari H. Nour, **Khan, M,** Ahmad Ziad Sulaiman, Tahira batool, Abdurahman H. Nour, Muhammad Mumtaz Khan, Faridah Kormin. In-vitro Anti-acetylcholinesterase and Antioxidant Activity of Selected Malaysian. *Asian Journal of Pharmaceutical and Clinical Research*. 7(3): 93-97. 2014.

Latest submissions.

Faridah Kormin, Farkaad A. Kadir, Rosli Mohd Yunus, **Khan, M**, Azhari Hamid Nour, Ade Chandra Iwansyah, Sharifah Bee Abd Hamid and Wageeh A. Yehye. Microwave Assisted Extraction; Phytochemical evaluation of Malaysian palm oil trunk epiphytes ferns. Chemistry Central Journal (Under review).

Conference Proceedings

Khan, **M.** Salah A.A.E., Khan, M.M. and Khan. N. Anti-Acetylcholinesterase activity of Piper sarmentosum by a Continuous Immobilized-enzyme assay. ***APCBEE Procedia, ELSEVIER*** 2, 199-204, 2012.

Khan, **M.,** Harun, N., A.H. Nour, Elhussein, S.A.A. 2013. *In Vitro* Antioxidant Evaluation of Fractions of Three Wild Malaysian Plants. ***Procedia Engineering ELSEVIER***, pp 29-36.

Khan, M. Elhussein, S.A.A. and Kambol, R.H. 2011. Inhibition of Acetyl cholinesterase by natural compounds of family Solanaceae from Malaysian Flora. International conference of chemical engineering and industrial biotechnology in conjunction with 25th symposium of Malaysian chemical engineers 2011 (ICCEIB-SOMChE 2011), from 28th Nov- 1st Dec 2011 at Hyatt Regency Kuantan, Malaysia.

Khan, **M**., A.H. Nour., Harun, N. and Elhussein, S.A.A. Evaluation of Antioxidant activity of four wild plant species from Malaysia to treat memory dysfunction by acetylcholinesterase inhibitory activity. 26th Symposium Of Malaysian Chemical Engineers (SoMChE), from 21-23 Nov.2012, UMS, Sabah Malaysia.

Khan, M. A.H. Nour, N. Harun and Elhussein, S.A.A. Antioxidant properties of mengkudu (Morinda citrifolia) leaves and fruit by beta carotene and TLC methods. 3rd National Conference on Postgraduate Research, from September 8-9, 2012, Universiti Malaysia Pahang, Kuantan, Malaysia.

Khan, M., A.H. Nour, A.Z. Sulaiman, N. Harun and Elhussein, S.A.A. Antioxidant and

anti-acetylcholinesterase activities of *Centella asiatica* (Pegaga) to treat memory dysfunction. I5th International Conference on Postgraduate Education (ICPS-2012), from December 17-19, 2012, UTM, Johor, Malaysia.

Harun, N., A.H. Nour, **Khan, M.** and S. Elhussein, S.A.A. Studies on antioxidant activity in *parkia speciosa* pods fractions. I5th International Conference on Postgraduate Education (ICPS-2012), from December 17-19, 2012, UTM, Johor, Malaysia.

Khan, M., A.H. Nour, A.Z. Sulaiman, Abdurahman H. Nour, and Elhussein, S.A.A. Non-edible part of *Capsicum annuum*- novel source of Acetylcholinesterase inhibition: Molecular docking and in vitro enzymatic studies. Malaysian Technical Universities Conference on Engineering and Technology (MUCET-2013), December 03-04, 2013, Kuantan, Pahang, Malaysia.

Khan, M., A.H. Nour, A.Z. Sulaiman, , Abdurahman H. Nour,, and Elhussein, S.A.A. Non-edible part of *Solanum melongena*- novel source of Acetylcholinesterase inhibition: Molecular docking and in vitro enzymatic and antioxidant studies. Industry-Academia Joint Initiatives in Biotechnology (CIA-Biotech-2013), December 05-07, 2013, Cameron, Pahang, Malaysia.

Muhammad Khan, Azhari H. Nour, Ahmad Ziad Sulaiman, Abdurahman H. Nour and Salah A.A Elhussein. Acetylcholinesterase inhibition by non-edible part of *Solanum tuberosum*: Molecular docking, antioxidant and in vitro enzymatic studies. Proceedings of Asian Federation of Biotechnology, Feb. 9-11, 2014, Kula Lumpur, Malaysia.

Khan, M, Azhari H. Nour, Ahmad Ziad Sulaiman, , Abdurahman H. Nour, Farhan Haidar Bangash. Phytochemical evaluation of three Solanum species to treat memory dysfunction: Molecular docking and in vitro enzymatic studies. BIOVISION, The World Wide Science Forum, June 05-06, 2014, Lyon France.