CHAPTER 1

1.0 INTRODUCTION

Traditional medicine is looked upon as an alternative or supplement to modern medicine and has made significant contributions to the health care of the world over the past decades. Various diseases such as diarrhea, skin problems, headache, fever, cough, wounds, hypertension, diabetes and rheumatism are treated with herbal medicine. Traditional medicines continue to be practiced by the community to treat disease and maintain health especially in remote areas where modern facilities are not readily available. Most of the medicinal plant species are collected from the wild, a few are being cultivated (Luseba*, et al.*, 2011)

Diabetes mellitus is a metabolic disorder in the endocrine system. This dreadful disease is found in all parts of the world and is becoming a serious threat to mankind health. There are lots of chemical agents available to control and to treat diabetic patients but total recovery from diabetes has not been reported up to this date. Alternative to these synthetic agents, plants provide a potential source of hypoglycemic drugs and are widely used in several traditional systems of medicine to prevent diabetes (Kavishankar, *et al.*, 2011)

The antidiabetic activity of herbs depends upon variety of mechanisms. The mechanism of action of herbal anti-diabetic could be grouped as Adrenomimeticism, which is the pancreatic beta cell potassium channel blocking cAMP (2nd messenger) stimulation (Marles & Farnsworth, 1996), the inhibition in renal glucose reabsorption (Eddouks, *et al.*, 2002), the stimulation of insulin secretion from beta cells of islets or and inhibition of insulin degradative processes (Pulok, *et al.*, 2006), the reduction in insulin resistance

providing certain necessary elements like calcium, zinc, magnesium, manganese and copper for the beta-cells regenerating and or repairing pancreatic beta cells (Pulok, *et al.*, 2006), the increasing the size and number of cells in the islets of Langerhans (Mohamed, *et al.*, 2006), the stimulation of insulin secretion (Esmaeili & Yazdanparast, 2004), the stimulation of glycogenesis and hepatic glycolysis (Miura, *et al.*, 2001), the protective effect on the destruction of the beta cells (Kim, *et al.*, 2003), the improvement in digestion along with reduction in blood sugar and urea (Krishnan, 1968), the prevention of pathological conversion of starch to glucose (Sepha & Bose, 1956), the inhibition of β-galactocidase and α-glucocidase (Sharma & Mujumdar, 1990), the cortisol lowering activities (Gholap & Kar, 2004), the inhibition of alpha-amylase (Hideaki, *et al.*, 2005) and the preventing oxidative stress that is possibly involved in pancreatic ß-cell dysfunction found in diabetes (Hideaki, *et al.*, 2005). Hence, the wide range of plant constituents could have different sites of action within the body, herbs exerts different mechanism of actions including the mechanism of actions of synthetic oral hypoglycemic drugs.

The constituents that comes under the category of polysaccharides, peptides, alkaloids, glycopeptides, triterpeniods, amino acids, steroids, xanthone, flavonoids, lipids, phenolics, coumarins, iridoids, alkyl disulphides, inorganic ions and guanidines are reported to have antidiabetic activity (Grover, *et al.*, 2002; Pulok, *et al.*, 2006).

Herbal therapy for diabetes has been followed all over the world successfully. Herbs were used to manage Type 1 and Type II diabetes and their complications. The herbs have been considered for their possible hypoglycaemic actions and the researchers have carried out some preliminary investigations. However, there are numerous other plants still await scientific inquiry, which have mentioned in the indigenous systems of health

care all over the world. A large number of plants, screened for their antidiabetic effect, have yielded certain interesting leads but till to date no plant-based drug has reached such an advanced stage of investigation or development as to substitute or reduce the need for the currently available oral synthetic drugs. However, the interest in herbal drug research continues with an expectation that some day or the other would be able to bring a safer and more effective compound with all the desired parameters of a drug that could replace the synthetic medicines (Kavishankar, *et al.*, 2011).

1.1 OBJECTIVE OF STUDY

1) To investigate the chemical composition of leaves and stems extracts of *Leptospermum flavescens*.

2) To measure the phenolic and flavonoid content and toxicity of the extracts.

3) To determine the antidiabetic properties of the extracts.

CHAPTER 2

2.1 INTRODUCTION OF *LEPTOSPERMUM FLAVESCENS*

2.1.1 Nomenclature and classification

Kingdom : Plantae

Subkingdom : Tracheobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Rosidae

Order : Myrtales

Family : Myrtaceae

Genus : *Leptospermum*

Species : *L. flavescens*

Figure 1.1 *Leptospermum flavescens*

2.1.2 Description

Leptospermum is a genus of about 83 species, all but three occurring in Australia. *Leptospermum flavescens* is one of the family of Myrtaceae. The other name or synonym name of *Leptospermum flavescens* is *Leptospermum polygalifolium*. The type specimen for *L. flavescens* was collected at Port Jackson in 1796. *L. polygalifolium* was known for many years as *L. flavescens*. It is commonly known as 'Gelam Bukit' or 'Cina Maki' in Malaysia. *L. flavescens* is an evergreen shrub growing to 3 m (9ft) by 3 m (9ft). It is hardy to zone 8. It is in leaf December to January. It is in flower from July to August. The flowers are hermaphrodite (have both male and female organs) and are pollinated by insects. It is noted for attracting wildlife. *L. flavescens* bark usually being

close and firm but soft, thick and flaky in some forms. The young stems at first have a close pubescence but usually become glabrous, with a conspicuous flange near the node and spreading and tending to curve around the stem. Leaves are sometimes aromatic, but not strongly, and are usually from 5 mm to 20 mm long, oblanceolate-elliptical to narrowly linear-elliptical, flat or with the margins recurved, and the tip often recurved with a soft or sharp point or, occasionally, a short pungent point. Flowers are usually white but may be greenish, off-white to pale pink and are usually 10 to 15 mm in diameter, occurring singly on modified shoots on the ends of very short or long branches, often in adjacent axils and on adjacent branches with new growth extending, mostly from branch ends, during or after flowering (Brickell & Zuk, 1997).

All forms of *L. flavescens* can be propagated easily from seed or from cuttings of medium hard wood which will strike fairly easily. Named cultivars such as 'Pacific Beauty' should only be propagated from cuttings as seedlings may vary significantly from the parent plants (Brickell & Zuk, 1997).

The plant prefers light (sandy), medium (loamy) and heavy (clay) soils, requires well-drained soil and can grow in nutritionally poor soil. The plant prefers acid and neutral soils. It cannot grow in the shade. It requires moist soil. The plant can tolerate strong winds but not maritime exposure (Brickell & Zuk, 1997).

Figure 1.2 Flower of *Leptospermum flavescens*

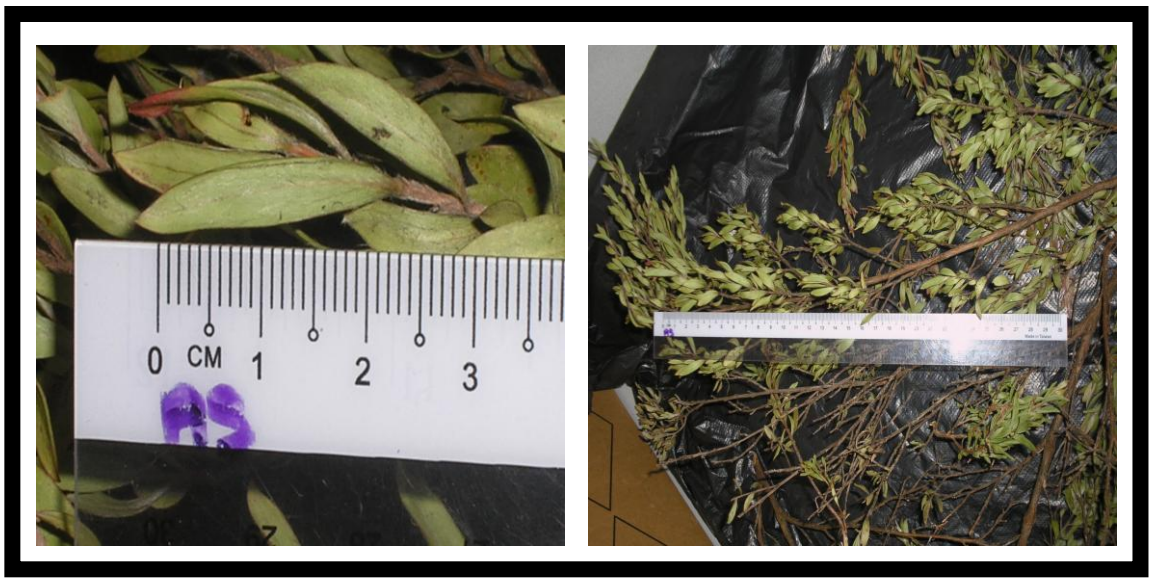

Figure 1.3 Leaves of *Leptospermum flavescens*

2.1.3 Medicinal Uses

L. flavescens, which has been used in Malaysia to stimulate appetite and relieve stomach disorders and menstrual discomfort also for diabetes (Riley, 1994). However, there is no scientific research has been carried out to measure the potential of *L. flavescens* for antidiabetic remedies.

Australia has unique native flora and produces honey with a wide range of different physicochemical properties and many Australian honeys have clinical potential. *L. flavescens* is one of the Australia unique native floras that produce honey with a wide range of different physicochemical properties. Honey derived from certain Australian flora possesses exceptional antibacterial activity. Non-hydrogen peroxide activity was detected and was most consistently seen in honey produced from *Leptospermum* spp. The non-hydrogen peroxide activity of *Leptospermum* honey samples increased, and this was greatest in samples stored at 25ºC. Among the *Leptospermum* species, *L. flavescens* (jelly bush) produced honey that was particularly high in antibacterial activity. Honey with non-peroxide activity is highly sought after in the medicinal honey market due to its potential clinical advantages. It was set up in 2009 after extensive research into the powerful properties of the Australian *L. flavescens* tree which produces the manuka honey (Irish, *et al.*, 2011).

2.1.4 Chemical Composition

An investigation of the chemical composition of the ethanolic extract of *L. flavescens* subspecies *flavescens* revealed the presence antimicrobial components including flavesone, leptospermone and isoleptospermone (Mustafa, *et al.*, 2003). Therefore, given the important biological activities of the volatile oils produced by *L. flavescens* species of the Myrtaceae (Cheng, *et al.*, 2009), the volatile oils produced by *L. flavescens*, presented as a major component the sesquiterpene nerolidol. The content of other components such as α-pinene, β-pinene, *γ*-terpinene, 1,8-cineole and terpinen-4-ol. The high concentrations of nerolidol in the oils of *L. flavescens* grown in Viçosa suggest that this oil can be used as food flavor additives. Besides, nerolidol is a sesquiterpene that has been tested as skin penetration enhancer for the transdermal delivery of therapeutic drugs (Cornwell & Barry, 1994) and as enhancer of bacterial permeability to antibiotics and antimicrobials (Byron & Johnson, 2003).

2.2 INTRODUCTION TO DIABETES MELLITUS

Diabetes Mellitus is a disease caused by the failure of the body function properly carbohydrate metabolism as well as changes in lipid and protein metabolism, thus contributing to hyperglycemia (increased blood sugar levels above normal), glycosuria (the presence of sugar in the urine), polyuria (the need to urinate frequently), polydipsia (always thirsty) and polyphagia (increase appetite). Hyperglycaemia caused by the failure of the pancreas secrete insulin insatiety, insulin resistance and the reduction of glucose by cells (Hashim, 2000). Diabetes is the category of chronic diseases which is still no cure and cause affliction in the patient. The increasing in cases of diabetes around the world becomes a major problem that must be addressed by society (O'Mathuna & Larimore, 2006). Global population world wide are at high risk of diabetes and in America about 15.6 million people have been diagnosed with diabetes (Rath, *et al.*, 2000).

2.2.1 History of Diabetes Mellitus

The term “diabetes” is derived from the Greek word which means “excessive urine production”, while the term "Mellitus" comes from Latin which means "sweet". Thus, the continuous increase of sugar in the blood is excreted through the urine and causes urine and blood on the sweetness of individuals with diabetes (Hashim, 2000).

2.2.2 Classification of Diabetes Mellitus

World Health Organization (WHO) classified diabetes mellitus into three types based on the etiology Diabetes Type I (Insulin-Dependent Diabetes Mellitus: IDDM), Diabetes Type II (Non-Insulin-Dependent Diabetes Mellitus: NIDDM) and Gestational Diabetes. Diabetes Type I caused by the failure of the pancreas produce insulin and without insulin the liver is unable to regulate blood glucose levels properly. Type I

diabetes usually occurs in children aged between 9-14 years and adolescents. Diabetes Type II is caused by a reduction in tissue sensitivity to insulin or resistance to insulin and it usually occurs in adults. Individuals who are obese face a higher risk of developing Diabetes Type II compared with normal individuals, while gestational diabetes occurs during pregnancy (Hashim, 2000).

2.2.3 Pathogenesis of Diabetes Mellitus

2.2.3.1 Diabetes Type I

i. Genetic factor

Genetic factors play a role in the development of Diabetes Type I, where more than one gene involved. Diabetes Type I is related with Histocompatibility Locus Antigen (HLA) and the study findings show higher HLA antigens in patients with diabetes compared with normal individuals. Genes involved in the discovery of HLA antigen found on the chromosome-6 and found more than one gene involved in the determination of HLA antigens such as B8 and B3. HLA antigen is a type of glycoprotein present in all human cell membranes and is believed responsible for detecting and destroying foreign molecule like bacteria and viruses. However, individuals with HLA antigens associated with diabetes do not guarantee the holder will have diabetes, but the risk of diabetes is higher (Olefsky, 1989).

ii. Autoimmune reaction

Type 1 diabetes mellitus (T1DM) is the result of immune-mediated destruction of insulin-secreting pancreatic beta cells. T cells that react to islet beta cells can contribute to the autoimmune response in diabetic patients and also play a part in self-tolerance in healthy individuals. The hormone insulin is usually not available directly in the diabetes type I. Changes in the function of HLA antigens normal detected as a factor

contributing to the autoimmune reaction resulting in the destruction of his own cells including the cells causes cells could not recognize its own beta cells of the host cell and more seriously, it is known to foreign molecules, further stimulate the production of antibodies to attack the beta cells causes insulin can not be produced (Olefsky, 1989).

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2.2.3.2 Diabetes Type II

i. Genetic factor

Diabetes Type II is influence by genetic factors which is high risk individuals if they have parents who suffer from diabetes type II. However, environment and lifestyle also as important role in the development of diabetes type II. Unhealthy lifestyle causes obesity increases the risk of developing diabetes (Olefsky, 1989).

ii. Insulin resistant

Insulin resistant characterized by reduced responsiveness to normal circulating concentrations of insulin is a common feature of almost all patients with type II diabetes. The presumed central roles of both peripheral and hepatic insulin resistance suggest that the enhancement of insulin action might be an effective pharmacological approach to diabetes. The severity depends on the degree of hyperglycemia and insulin resistance, it occurs in the liver tissue and muscle tissue caused by changes in insulin receptor found on the surface of the liver tissue and muscle tissue. Insulin resistance in the liver causing excessive glucose production and glucose consumption low during full (Campbell & Reece, 2004).

Gestational diabetes is carbohydrate intolerance resulting in hyperglycemia of variable severity with onset or first recognition during pregnancy. It does not exclude the possibility that the glucose intolerance may antedate pregnancy but has been previously

unrecognized. The definition applies irrespective of whether or not insulin is used for treatment or the condition persists after pregnancy. Problem of resistance to insulin causes insulin receptors expressed less then give effect to the dispatch system signals (Hashim, 2000).

2.2.4 Complication of Diabetes Mellitus

Diabetes is a complex condition which can result in long term complications. There is no such thing as 'mild' diabetes. Whether diabetes is managed by healthy eating and physical activity alone or in conjunction with tablets and or injections, poorly controlled diabetes will cause damage to your body. High blood glucose levels over a period of time can damage the small and large blood vessels and nerves. The most common complications that occur in people with diabetes include:

- Cardiovascular disease contributed to death of adult diabetes in which the frequency of heart attacks for individuals with diabetes are three times higher than normal individuals (Eastman, *et al.*, 1993).
- Diabetic neuropathy is a complication of diabetes, where almost 50% of patients with diabetic neuropathy diabetic disease resulting from continued improvements in blood sugar. High risk feet have lost feeling (peripheral neuropathy) and poor blood flow (peripheral vascular disease). People who have had a foot ulcer or amputation in the past have high risk feet. Feet with calluses or deformities like claw toes also have increased risk if poor feeling and or decreased blood flow are also present (Eastman, *et al.*, 1993).
- Diabetic rethinopathy causes a reduction in the sense of sight in about 95% of patients with type II diabetes caused by obstructions in blood vessels of the retina is almost 2 % of patients with diabetes become blind (Davis, 1992).

- Diabetes causes kidney function, the study showed that about 10% of patients with diabetes experience kidney failure (Herman, 1990).

2.2.5 Diabetes Treatment

Treatment for type I diabetes existing at present is the treatment of insulin injections while type II diabetes is by controlling diet and daily physical activity. Many people with diabetes successfully control blood sugar levels through daily exercise and a healthy diet and some people with diabetes need medication. Prevention of diabetes is very important because at present there is no drug that could cure diabetes completely. Diabetes treatment involves two steps of diabetes prevention. First selection of prevention for a healthy diet and physical activity which is can reduces the risk for high risk individuals, while the second selection of prevention for initial examination to help individuals who have been diabetes diagnosis (Campbell & Reece, 2004).

2.2.6 Mechanism of glucose regulation in normal individual

Regulated blood sugar level should be regulated so that it does not exceed the normal level (90 mg/100 ml), thus contributing to hyperglycemia. The pancreas is the organ responsible for controlling blood sugar levels. And alpha beta cells in the pancreas produce insulin, respectively, and glucagon, a hormone involved in the regulation of dominant sugar levels. Both these hormones act to fight and balance of glucose metabolism in the body and prevent hyperglycemia. Normal levels of blood glucose is stable, example 90 mg/ml, even after eating and in fasting conditions (Campbell & Reece, 2004).

2.2.7 Mechanism of insulin reaction

Under normal circumstances, insulin lowers blood glucose levels by promoting storage of glucose into glycogen in the liver, inhibit glucose production, it also acts as a modulator of protein synthesis and triglyceride synthesis in adipose tissue.

After eat a meal, the system of body digests carbohydrate into glucose and absorbed into the blood cause increase blood sugar. Receptors on beta cells to detect the increase in glucose, activates receptors on beta cells, thereby stimulating the beta cells to secrete insulin into the blood. Insulin binding to the receptors found on the target system, the dispatch of subsequent cells activates a signal and its effect lowers the glucose concentration to promote storage of glucose as glycogen and promote greater utilization of glucose by the cells of the body to carry out daily activities. After blood glucose levels return to normal levels, stimulation of the beta cells also reduced the production of insulin (Unger, *et al.*, 1978).

2.2.8 Mechanism of glucagon reaction

Together with insulin glucagon is important hormone in the mechanisms regulating blood sugar. It is secreted by cells found in pancreatic alpha where it stimulates the production of cyclic adenosine monophosphate, the carrier signal in response production and gluconeogenesis and glycogenolysis when there is a stimulus decrease blood sugar levels. Like insulin, glucogon production by the cells decreased due to alpha stimulation in the cells decreased (Gorich, *et al.*, 1976).

2.2.9 Mechanism of glucose regulation in diabetes patient

The main glucose production in the liver through glycogenesis and glycogenolisis insulin and glucagons play a role in stimulating and inhibiting the process based on the

stimulus. Insulin has a retarding effect on glycogenolisis while it stimulates glycogenesis. For patients with type II diabetes, insulin resistance causes the dispatch system, the signal can not be implemented well, resulting in inhibition glycogenolysis also reduced, increasing the activity of GP continuing to further encourage the production of glucose in the liver that contribute to hyperglycemia (Pfeifer, *et al.*, 1989).

2.3 GLYCOGEN PHOSPHORYLASE (GP)

2.3.1 Introduction of enzyme

Excess glucose is stored in the liver as glycogen through glycogenesis. The broken down of glycogen into glucose when the body's cells need energy to carry out the process of breaking of bonds glycogenolisis glycosodic irreducible glycogen molecules. Glycogenolisis stimulated by the glycogen phosphorylase (GP). GP is homodimeric with 846 residues per subunit that exists in two forms that can switch between each other, (interconvertible) of GP_b (less active, dephosphorylation form) and GP_a (more active, phosphorylation form) and GP conversion is influenced by phosphorylation and binding action on the site alosteric effect or enzymes (Rath, *et al.*, 2000). Binding inhibitor at the site alosteric change its shape and function of GP active site caused glycogen GP can not bind to the active site of GP and stabilization by the inhibitor alosteric GP_b causing glycogen breakdown to glucose response by inhibited GP_a (Campbell & Reece, 2004). GP has at least five active site of ser 14 phosphate recognition site (Ser 14 phosphate-recognition site), site activator AMP (AMP activator) and inhibitors of glucose-6-phosphate (glucose-6-P inhibitory allosteric site), catalytic site (catalytic site) that binds glycogen and glucose-1-phosphate, site inhibitors and glycogen storage site (Oikonomakos, *et al.*, 2000).

2.3.2 Mechanism of enzyme reaction

Regulation of GP activity helps regulate the metabolism of glucose by the liver so that blood sugar levels are controlled. GP activity is regulated by covalent modification by phosphorylation and binding reaction at the site alosteric effector enzymes. GP stimulated by $_C$AMP and inhibited by glucose-6-phosphate. GP only be binding on glycogen molecules in the active state, GP_a. If there is no inhibition on glycogenolisis, GP becomes active and promotes the production of glucose in the liver.

The series of enzymatic reaction with GP reaction initiated by the system signal messenger, $_{C}$AMP. $_{C}$AMP is actually derived from ATP through the action of enzyme previously associated edenylate cyclise by G protein. $_{C}$AMP production increased, causing more $_{C}$AMP binding to the regulatory subunit of protein kinase A (PKA), thereby removing the effect of regulatory subunit of PKA inhibition on to active PKA. Phosphorylase kinase activation by PKA is activated by phosphorylation of action. Protein kinase is activated GP_b phosphorylated to GP_a. GP_a glycogen molecules react with ties decided glycosidic $\alpha 1,4$ at the end of irreducible glycogen molecule, producing free glucose for the cell (Hashim, 2000).

The liver is largely responsible for unrestrained glucose production through increased rates of gluconeogenesis and glycogenolisis. Potential drug targets that modulate these processes include the glucagon receptor (antagonists), glycogen phosphorylase (inhibitors), and other rate-controlling enzymes such as glucose-6-phosphatase and fructose-1, 6-bisphosphatase (inhibitors). Defective glucose-stimulated insulin secretion by pancreatic islet β-cells could be alleviated with recombinant glucagon-like peptide 1 (GLP-1) or agonists of the GLP-1 receptor. Alternatively, decreased GLP-1 clearance can be achieved with inhibition of dipeptidylpeptidase IV (DP-IV). To reduce insulin resistance, enhanced insulin action in liver and muscle (and fat) might be achieved with small-molecule activators of the insulin receptor or inhibitors of protein tyrosine phosphatase (PTP)-1B. Other potential drug targets in the insulin signaling pathway are discussed in the text. The development of anti-obesity agents that produce reduced appetite and/or increased energy expenditure will also lead to effective treatment (prevention) of type II diabetes (David Moller, 2001).

2.3.3 Enzyme inhibitor

The GP inhibitor stabilises the GP_b through the action of dephosphorylation, binding of heterocyclic compounds such as glucose and caffeine. Glucose binds to the active site of GP, while caffeine binds to alosteric site where this binding inhibits the activity of GP. The GP inhibitor includes glucose analog that competes with the glycogen molecule to bind the enzyme active site and AMP binding sites. Studies have shown that the binding of caffeine on alosteric site, lower affinity binding of $_C$AMP and compete with glucose-1-phosphote to bind to the enzyme active site (Fang & Nuttal, 1997). Scientists have found the inhibitor indole-2-carboxyamide where response inhibition was almost the same as caffeine and glucose action where it is binding at the active site permanently change activities GP_a. The indole-2-carboxyamide have been inhibiting effect on hypoglycemia and potential use as antidiabetic agent (Rath, *et al.*, 2000).

2.4 INTRODUCTION TO LIQUID CHROMATOGRAPHY MASS SPECTROMETRY COMBINED WITH MASS SPECTROMETRY (LCMS/MS)

Liquid chromatography (LC) combined with mass spectrometry (MS) is a powerful tool for qualitative and quantitative analytics of organic molecules from various matrices, and the use of this hyphenated technique is very common in bio-analytical laboratories. In this study, LCMS/MS methods and the required sample preparation applications were developed for detection of bioactive compounds in crude extracts of *L. flavescens* studies. The LCMS/MS techniques have clearly become the most widely-used tool in analyzing the bioactive compounds of plants and their metabolites (Korfmacher, 2005; Tolonen, *et al.*, 2009). Typical steps in these analyses consist of sample preparation, chromatographic separation, mass spectrometric detection and data processing. The sample preparation is an important step in all analyses, and especially when analyzing biological fluids (Kataoka, 2003; Nováková & Vlˆcková, 2009). The analytic must be extracted from the sample matrix components and other impurities that may reduce the specificity of detection or interfere with the mass spectrometric ionization process by suppression or enhancement. A commonly used sample preparation method in LCMS/MS analysis is still traditional liquid-liquid extraction (LLE), although the method is time-consuming and produces great amounts of solvent waste(Nováková L. & Vlˆcková H., 2009). Another commonly used method, solid phase extraction (SPE), uses a sorbent material containing a solid phase and a flowing liquid phase to isolate a compound or certain type of compounds from a liquid sample. The sample is loaded into cartridge, undesired compounds are washed away, and the analytics are eluted with a different solvent (Chen, *et al.*, 2008). Numerous SPE cartridges containing different packing materials are available and can be used for extracting acidic, basic and neutral compounds from different kind of matrices and also use the ion exchange principle

(Fontanals, *et al.*, 2005). It is worth noting that when using extraction methods the discarding of metabolites with different recoveries can occur and thus modify the metabolic profile of the study compound. Therefore, in the case of metabolite profiling, it is recommended that unspecific sample preparation methods such as protein precipitation with acetonitrile or methanol rather than SPE or LLE be used.

For metabolic studies the most common ionization methods in LCMS/MS applications are Electro Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI). The ion sources are referred to as API sources, as the ionization does not take place in a vacuum but in atmospheric pressure. ESI is suitable for almost all drug-like molecules with at least one easily ionizable functional group(Cech & Enke, 2001), whereas for steroids and other less polar compounds, APCI is often utilized (Leinonen, *et al.*, 2002). Of these two methods, APCI is generally less susceptible to matrix effects (Dams, *et al.*, 2003 ; Niessen, *et al.*, 2006). The negative ionization polarity is considered more specific, whereas the positive ionization mode is more vulnerable to ion suppression (Antignac, *et al.*, 2005; Niessen, *et al.*, 2006). In ESI, the analyte is ionized within the solution and thus the chemistry of the liquid phase has a great impact to ionization efficiency. The solution containing the analyte is directed through the high voltage capillary (2 – 5 kV), where the capillary voltage gathers the oppositely charged ions to the sides of the capillary and thus the droplets spraying from the capillary are excessively charged with the same polarity as that in the capillary. The liquid flow and gas flow parallel to the capillary aid the formation of droplet mist. The droplets diminish by means of vaporization (aided by drying gas flow) until the repulsion forces of the similarly charged ions are larger than the cohesive strengths of the surface tension, when the individual ions vaporize to gas phase (ion evaporation method, IEM). In the charge residue model (CRM) the vaporization of the solvent continues until only single

ion droplets remain. The IEM is believed to be dominant with the small molecules, whereas in the case of large molecules, such as proteins, CRM occurs (Kebarle, 2000). The ion suppression or enhancement by the co-eluting component may occur due its effect to droplet formation or the availability of charge (proton), or co-precipitation of the analyte from the spray as a neutral molecule (Jessome & Volmer, 2006; Liang, *et al.*, 2003 ; Tang, *et al.*, 2004).

From the MS analyzer point of view, quadrupole instruments are most used in quantitative analysis due to their high sensitivity, good specificity and high linear range, whereas TOF instruments are used in screening type analyses, such as profiling of unknown metabolites, because they offer high sensitivity wide mass range scan with high mass resolution (>40 000 FWHM) and high mass accuracy. However, modern time-of-flight mass spectrometers could be used for quantitative work as their linear range has significantly improved recently due to the introduction of dynamic range enhancement systems (Williamson & Bartlett, 2007).

CHAPTER 3

3.1 EXPERIMENTAL MATERIAL

The fresh herbs samples of *Leptospermum flavescens* was obtained from Genting Highland, Pahang, Malaysia as experimental material (Figure 1.1). The authentic taxonomic identification was confirmed by plant taxonomist, Profesor Dr. Ong Hean Chooi from Institute of Biological Science, University of Malaya. In this study, only the leaves and stems of the *Leptospermum flavescens* herbs were used separately. The selected herbs were ensured to be free from any disease. The herbs were washed and cut into small pieces for efficient drying process. The herbs then dried in room temperature for 72 hours. Each dried samples were ground into fine powder and it was stored to further study.

3.2 PREPARATION OF EXTRACTS

For the plants extraction, 20 g of herbs samples powder were extracted using hexane, chloroform, and water. 200 ml of hexane was added to 20 g of herbs samples in a conical flask. The hexane extract was prepared by extracting the leaves powder and stems powder separately in water bath for 4 hours at 37 ºC. Then hexane extract were placed in the incubator shaker at room temperature (27 ºC), with the rotation speed of 220 rpm for 72 hours. After 72 hours, each of the extract was filtered using Whatman filter paper. The filtered extracts were then evaporated to a concentrated crude extract using a rotary evaporator at 37 ºC. The concentrated crude extracts were then transferred into a freeze drying flusks which was frozen at -80 ºC for five days then started become to dried powder extracts used by freeze drying machine (Figure 8.10). The dried powder extracts were weighted and then kept at -20 ºC until for the next experiment tests used. The above procedures were repeated for both chloroform and water extraction.

3.3 SEPARATION OF CHEMICAL COMPOUNDS

Chromatography is a method that can be used in separation of two or more compounds or ions between two phases. Although there were many different variations of chromatography, it had the same principle.

A) Thin Layer Chromatography

In order to separate the chemical compounds from the crude extract, thin layer chromatography (TLC) was used. TLC was a solid form of chromatography where the stationary phase was normally polar absorbent and the mobile phase can be a single or combination of solvents. It was a quick and cheaper microscale technique. These absorbents created very polar materials and the more polar the molecule to be separated, the stronger the attractive force to the stationary phase. The polar stationary phase was more strongly attracted polar molecules (Harborne, 1973).

Solvent was poured into the beaker (TLC chamber) to a depth of just less than 0.5 cm and it was covered with watch glass. Chamber was allowed to stand and saturated while preparing for TLC plate. TLC plate was prepared and a line was drawn across to mark as ‘original’ of the paper. Next , sample was spotted on the ‘origin’ with capillary tube and the prepared TLC plate was placed in the developing beaker and it was covered with watch glass. Plate was removed from the beaker after the solvent has risen to ¾ of the plate. Then, the solvent front was marked immediately. The plate was placed in the fume cupboard to allow the solvent to evaporate. After it sprayed with different reagent, the colored spot formed and it marked again. R_f value calculated by using below formula (Harborne, 1973):

$$R_f = \frac{\text{Distance traveled by the compound, X (cm)}}{\text{Distance traveled by the solvent, Y (cm)}}$$

The R_f value was the distance of compound moved in chromatography relative to the solvent front. It was obtained by measuring the distance from the origin to the centre of the spot produced by the substance, and this was divided by the distance between the origin and the solvent front. In identifying a plant constituent, once it had been isolated and purified, it was necessary first to determine the class of compound and then to find out which particular substance it was within that class. The class of compound was usually clear from its response to color tests, its solubility and R_f properties and its UV spectral characteristics (Harborne, 1973).

B) High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) was a highly improved form of column chromatography. It was forced under high pressure and that makes it much faster. Smaller particle size was used for the column packing material that gave much higher surface area for interaction between the stationary phase and molecules passing through it. Usually, detection of the eluted compounds was one of the most difficult problems, but in HPLC the detection method was highly automated and very sensitive (Lima, *et al.*, 2007).

The major parts of HPLC were solvent reservoir, pump or solvent delivery systems, sample injector, column, detector, data analysis and waste bottle. HPLC grade solvent was used because of its purity. Solvent, mobile phase and test samples had to be filtered with 0.45 µm micro filter for separation of an impurity which can damage column and system.

Pump was used to force the eluent through the column and detector. If a non-polar stationary phase (C18) used with polar mobile phase (acetonitrile:water), non-polar solute was retained more and polar solute was eluted. The retention time was varied depending on the interaction between the stationary phases and molecules that have been analyzed and the solvents that used (Lima, *et al.*, 2007).

i. Preparation of high performance liquid chromatography (HPLC) for crude extract of *L. flavescens*.

In this study, an isocratic method was used for crude extract sample of hexane, chloroform and water from parts of leaves and stems of *L. flavescens*. HPLC analysis was performed using Shimadzu chromatograph equipped with an automatic injector,

column oven and UV detector (Figure 8.11). Isocratic was a constant composition of mobile phase. The HPLC method applied was a modification of that reported by (Lima, *et al.*, 2007). 10 to 20 µL of crude extract samples were separated within a total time of 60 minutes and flow rate of 0.8 ml per minutes.

The peaks were detected at 254 nm. The results was then compared with HPLC chromatogram of the *L. flavescens* leaves and stems extract by using different solvents (Lima, *et al.*, 2007) in order to identify the chemical compound in *L. flavescens*. Acetonitrile-water was used as mobile phase. Acetonitrile-water (5:95 v/v) was in pump A and Acetonitrile-water (9:1 v/v) was in pump B. The distilled water was filtered by using 0.45 µm membrane filter for 3 times before it mixed (Lima, *et al.*, 2007).

Degasser or sonicator was used to get rid of air bubble of the solvent before it pumped into the HPLC machine. pH measurement was also important in HPLC solvent system because it can change the hydrophobicity of the analyte. The pH of the mobile phase must be >2 and <10. For this reason, we must use a buffering agent such as sodium phosphate to control the pH. The buffers can control the pH and neutralized the charge to improve the chromatography. The setting of the Shimadzu HPLC machine was carefully done because all the HPLC equipments were very sensitive and easily broken (Lima, *et al.*, 2007).

After the samples were filtered with 0.45 µm microfilter, their solubility was tested by using mobile phase, and only test sample that was dissolved in the mobile phase proceed in the next step. We have to wait for about 20 to 30 minutes to make sure that the pressure was stable before the injection started. Injector was a syringe that was used to inject the sample into the eluent stream through a rubber septum. The syringe must be rinsed with deionized water before being used to prevent contamination. Chromatogram

can be observed through computer that was connected with HPLC system (Lima, *et al.*, 2007). When we observed the HPLC chromatogram, retention time played an important role. Retention time was the time that taken for a particular compound to travel through the column of the detector. Time was measured from time at which the sample was injected to the point at which the display showed a maximum peak height for that compound. After the HPLC was run about 30 minutes, the mobile phase for washing had to prepare. Usually if the buffer was used as a mobile phase, 100 % of deionized water was used for 30 minutes (for both pumps) and continued with 70:30, methanol or acetonitrile: deionized water. If no buffer was used, only 70:30, methanol or acetonitrile: deionized water was used for washing (Lima, *et al.*, 2007).

ii. Preparation of high performance liquid chromatography (HPLC) for gallic acid (GA), tannic acid (TA) and quarcetin (Q) standard.

All the test samples were filtered with 0.45 µm micro filter. Solubility test was done to the filtered samples and only dissolved samples were injected into HPLC machine. Gallic acid, tannic acid and quarcetin at 0.01 g/ml concentration were prepared freshly on a daily basis. 10 µL of injection volume were taken from each test sample and its flow rate was 0.8 ml/min. All the samples were separated with two solvent systems: 0.05 % trifluoroacetic acid (TFA) in water and 0.05 % TFA in acetonitrile. The peaks were detected at 228 nm and were identified by standard substances (Lima, *et al.*, 2007).

C) Liquid chromatography mass spectrometry combined with mass spectrometry (LCMS/MS)

i) Sample preparation

These leaves and stems of *L. flavescens* separately were extracted with hexane, chloroform and water to the concentration of 0.1 g/ml. For the LCMS/MS experiments, final concentrations of leaves hexane were 0.1–10 µg/ml in 2 ml dichloromethyl + 1 ml acetonitrile + 2 ml methanol. Leaves chloroform were 0.1–10 µg/ml in 2 ml acetonitrile + 3 ml water. Leaves water were 0.1–10 µg/ml in 5 ml methanol. Stems hexane were 0.1–10 µg/ml in 2 ml dichloromethyl + 1 ml acetonitrile + 2 ml methanol. Stems chloroform were 0.1–10 µg/ml in 2 ml acetonitrile + 3 ml water. Stems water were 0.1–10 µg/ml in 5 ml methanol. Methanol, dichloromethyl and acetonitrile were of HPLC grade and supplied by Merck AG, Darmstadt, Germany. Each 0.01 g sample weighted and diluted into appropriate solvents then sample was filtered with 0.22 µM nylon filter and the volume injection is 20 µL.

ii) LCMS/MS procedure

LCMS/MS analyses were performed using full scan with MS/MS data collection. Column: Phenomenex Aqua C18-50 mm x 2.0 mm x 5 µM. Buffer A was Water with 0.1 % formic acid and 5 mM ammonium formate. Buffer B was Acetonitrile with 0.1 % formic acid and 5 mM ammonium formate. Rapid screening at 15 minutes runs time. AB Sciex 3200QTrap LCMS/MS Gradient run programmer: 10% A to 90 % B from 0.01 minutes to 8.0 minutes hold for 3 minutes and back to 10 % A in 0.1 minutes and re-equilibrated for 4 minutes. Pre-run equilibration time: 1.0 minutes. Electro Spray Ionization (ESI) source was used in negative mode in the study.

3.4 DETECTION AND ISOLATION OF CHEMICAL COMPOUND

After the compound has been isolated and purified, we have to determine the class of compound to take a further test. The detection of chemical compound of TLC plates was carried out by observing under visible light, under UV light and spraying with Dragendorff, Vanilin and Anisaldehyde reagent. The identification of the isolated compound within the class depends on measuring other properties when compared to the literature (Harborne, 1973).

A) Visible light

Under visible light, the coloured of the chemical compound can be seen clearly. The chemical compound that appeared was a compound that contains pigments that absorb lights. TLC chromatograms of each sample were observed under visible light. Available bands on the plate were mark and R_f value were calculated. Green colour band indicate the presence of chlorophyll in the sample while carotene gives red, orange or yellow bands (Harborne, 1973).

B) Ultra Violet Light

Under ultra violet (UV) light, the aromatic compounds can be identified. Aromatic compounds represent phenolic, quinine and flavonoid compounds. TLC chromatograms were observed under ultra violet light in the dark room by using UV lamp to spot for fluorescent band and R_f value of each band was calculated. Fluorescent green band indicated presence of aromatic compound in the sample (Harborne, 1973).

C) Dragendorff Reagent

Apart from using UV light, Dragendorff reagent can be used to detect the chemical compounds that may present. If the chemical compounds showed orange colour, it

indicated the presence of alkaloids. Dragendorff reagent was prepared by mixing 5 ml of Solution A [1.7 g basic bismuth nitrate in 100 ml H_2O-HOAc (80:20)] with 5 ml Solution B (40 g KI in 100 ml distilled water), 20 g acetic acid and 70 ml distilled water were added. Dragendorff reagent was used to spray the TLC chromatograms to determine the presence of alkaloid compound in the samples. Then the sample was aired dry. Orange spot on the plate indicated the presence of alkaloid and choline in the sample, koumarin gave an orange spot. R_f value for the alkaloid compound was calculated and recorded (Harborne, 1973).

D) Vanillin Reagent

Another reagent that can be used to detect the chemical compound was Vanillin reagent. It was sprayed onto the TLC plate and it heated at about 100 °C. If the purple colour appeared, the compound was indicated as terpenoid but if the green colour appeared, it showed the presence of phenol compound. Vanillin reagent was prepared by mixing 5 % ethanol sulfuric acid with 1 % ethanol vanillin. TLC chromatograms were spray with Vanilin reagent. Then they were heated in a hot plate at about 100 °C. Terpenoid gave purple band while phenolic compound gave red or other colour spot. R_f value of each coloured spot was calculated and recorded (Harborne, 1973).

E) Anisaldehyde Reagent

Anisaldehyde reagent is one of the reagents that can be used to detect the chemical compound. The TLC plate is sprayed with about 10 ml, heated at 100 °C for 5-10 minutes, and then evaluated in visible or UV-365 nm. The reagent has only limited stability and is no longer useable when the colour has turned to red-violet. This reagent was used to detection of terpenoids and saponins. R_f value of each coloured spot was calculated and recorded. 0.5 ml Anisaldehyde reagent was prepared by mixing 10 ml

glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid (Harborne, 1973).

3.5 DETERMINATION OF TOTAL PHENOL CONTENT

Phenolic compounds have been found to be strong antioxidants against free radicals and they were also reported to be an antioxidant activities may be useful for meal planning in type 2 diabetes. They could contribute to sustain plasma antioxidant level because antioxidants present in plants and herbs prevent the development of vascular diseases seen in type 2 diabetes (Büyükbalci & Nehir, 2008). Total phenol content has been expressed in the terms of gallic acid equivalents, catechin equivalents, tannic acid equivalents and caffeic acid equivalents. Measurement of total phenolic content indicated the amount of phenolic compounds presented in the plant samples. In this study gallic acid was used as a standard to measure the phenolic compound from the leaves and stems extract of *L. flavescens*.

500 µL samples or standard solution was added with 5 ml Folin Ciocalteu reagent (1:10 is diluted with distilled water) and 4 ml of 1 M $NaCO_3$ solution. The reaction mixture incubated in water bath at 45 °C for 15 minutes. The absorbance was measured at 765 nm with spectrophotometer. Standard curve was prepared by diluting 0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/ml gallic solution in methanol: water (50:50, v/v). The total phenol was calculated as gallic acid equivalents mg/g, dry mass (Adedapo, *et al.*, 2008).

3.6 DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid (TF) concentration was determined using a colorimetric assay (Meyers, *et al.*, 2003; Tsantili, *et al.*, 2010). The absorbance of the solution was measured and a blank at 510 nm using a spectrophotometer. The results were expressed as mg of quarcetin equivalents (QE) per g dried weight (mg $^{-1}$ dry weight). The total flavonoid content of the samples was determined using a modified colorimetric method described previously by (Zhishen, *et al.*, 1999) and used quarcetin as a standard. Extracts or standard solutions (250 mL) were mixed with distilled water (1.25 mL) and 5 % $NaNO_2$ solution (75 mL). After standing for 6 minutes, the mixture was combined with 10 % $AlCl_3$ solution (150 mL). 1 M NaOH (0.5 mL) and distilled water (275 mL) was added to the mixture 5 minutes later. The absorbance of the solutions at 510 nm was then measured. The results were expressed as mg quarcetin equivalents (mg, quarcetin/g extract).

3.7 BRINE SHRIMP LETHALITY ASSAY (BSLA)

Brine shrimp lethality bioassay was carried out to investigate the cytoxicity of plant extracts. The method has also been applied to plant extracts in order to facilitate the isolation of biologically active compound. The technique was easily done and the cost was cheaper, and it utilized small amount of test material. This in vivo lethality test has been successfully employed for bioassay-guide fractionation of active cytotoxic and antitumor agents from medicinal plants. In this study, BSLA have been used to measured the toxicity of the leaves and stems crude extract of *L. flavescens* (Olaleye, 2007).

38 g sea salt per liter of water was prepared to make 'sea water'. The sea water was prepared in small tank and shrimp eggs were added to one side of the divided tank and were covered with aluminium foil. The lamp above the uncovered side will attract hatched shrimp through perforations in the dam. The shrimps were allowed 2 days to hatch and mature as nauplii. Vials for testing were prepared; test initially at 1000, 100, 10 µg/ml; 3 vials were prepared at each concentration for a total of 9 vials; 20 mg of sample weight and 2 ml of water (20 mg/2ml) were added; from this solution 500, 50, 5 µL was transferred to vials corresponding to 1000, 100 or 10 µg/ml respectively (Olaleye, 2007).

After 2 days when the shrimp larvae were ready, the sea water was added to each vial, 10 shrimp per vial (30 shrimp per dilution) were counted and the volume was adjusted with sea water to 5 ml/vial (shrimp can be used 48-72 hours after the initiation of hatching). After 72 hours, they should be discarded. 24 hours later, they were counted and the number of survivors was recorded. The data was analyzed with Finney computer programme to determine LC_{50} values and 95 % confidence (Olaleye, 2007).

3.8 ANTIDIABETIC ACTIVITY BIOASSAY – HYPERGLYCEMIA ASSAY

Microplate reader (Bio-Rad) based on the published methods have been used to monitor the enzymatic inhibitor of Phosphorylase activity (Martin, *et al.*, 1998). Each compound was dissolved in DMSO and diluted at different concentrations for IC_{50} determination. Glycogen phosphorylase a were added into the 100 µL buffer with compounds dissolved in containing 50 mM Hepes (pH 7.2), 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM glucose-1-phosphate and 1 mg/ml glycogen in 96-well microplates (Costar). After the addition of 150 µL of 1 M HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green, reactions were run at 22 ºC for 30 minutes and then phosphate absorbance was measured at 655nm (Chen, *et al.*, 2007). Glycogen phosphorylase an activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate. In this study, caffeine was used as the standard in the glycogen phosphoryase (GP) inhibitory assay. The ability of the Caffeine acted as GP inhibitor to inhibit the process of glycogen degradation and reduce hepatic glucose production (HGP). Various concentration of caffeine were tested; 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml. The GP inhibitory activities of hexane, chloroform and water extracts from the leaves and stems of *L. flavescens* were determined (Table 4.20 & Figure 4.23). All the crude extract with 5 different concentrations was also tested by using GP inhibitory activity to measure the percentage inhibition of GP activity.

3.9 ANIMAL STUDY

This study was approved by the animal care and use committee (ACUC), Faculty of Medicine, University of Malaya [Ethic No.; ISB/03/03/2010/JM(R)].

3.9.1 Determination of oral glucose tolerance test (OGTT)

The oral glucose tolerance test (Shirwaikar, *et al.*, 2006) was performed in overnight fasted (18 h) normal rats. Rats divided into six groups, each consisting of six rats were administered 0.9 % (w/v) saline, Glipizide dose at 5 mg/kg (Chitra, *et al.*, 2010), *L. flavescens* water extracts doses at 0.2 g/kg and 0.5 g/kg, respectively. Glucose concentration at 3 g/kg was fed by oral gavage administration 30 minutes after the administration of extracts (Taiwo, *et al.*, 2009). Blood was withdrawn from the retro orbital sinus under ether inhalation at 0, 30, 60, 90 and 120 minutes of glucose administration and glucose levels were estimated using glucose oxidase–peroxidase reactive strips and a glucometer (Accuchek, Roche Diagnostics, USA).

3.9.2 Determination of acute toxicity of *Leptospermum flavescens*

Healthy overnight fasted adult male Sprague–Dawley (SD) rats of starved overnight were divided into five groups, each group consisting ten rats. Group 1 was fed by oral gavage administration with the leaves water extracts in dose level of 0.2 g/kg body weight, Group 2 was fed by oral gavage administration with the leaves water extracts in dose level of 0.5 g/kg body weight, Group 3 was fed by oral gavage administration with the stems water extracts in dose level of 0.2 g/kg body weight, Group 4 was fed by oral gavage administration with the stems water extracts in dose level of 0.5 g/kg body weight, Group 5 was fed by oral gavage administration with the sterile water as a control normal group. The acute toxicity study was carried out according to OECD

guidelines–425. The animals were observed continuously for 2 hours under the following profiles (Barik, *et al.*, 2008).

(I) Behavioral profile: Alertness, restlessness, irritability, and fearfulness.

(II) Neurological profile: Spontaneous activities, reactivity, touch response, pain response and gait.

(III) Autonomic profile: Defecation and urination.

After a period of 24 h, 72 h and 14 days they were observed for any lethality or death.

3.9.3 Experimental induction of non-insulin dependent diabetes mellitus (NIDDM)

Overnight fasted adult male Sprague–Dawley (SD) rats weighing (150 g to 200 g) purchased from the animal house, University Malaya Medical Center (UMMC), Petaling Jaya, Malaysia were used in the experiments. They were kept for 2 weeks in our laboratories before the experiments for acclimatization to the laboratory conditions and fed with standard pellet diet and water (Figure 8.7). All animals were housed in individual cages in a room controlled for temperature (23 ± 1 °C), humidity (50 ± 4 %) and light (08:00 h – 20:00 h), and were maintained on a laboratory diet, rats were housed one per cage. Prior to experimental treatments, animals were fasted overnight but were allowed free access to water. Six animals were used for each group of study.

NIDDM was induced in overnight fasted adult male Sprague–Dawley (SD) rats weighing (150 g –200 g) by a single intraperitoneal injection of 120 mg/kg Alloxan monohydrate (Figure 8.8 and Figure 8.9) (Chitra, *et al.*, 2010; Mukhtar, *et al.*, 2004). Hyperglycemia was confirmed by the elevated glucose levels determined at 72 h. Animals with blood glucose level more than 150 mg/dl or 8.3 mmol/L were considered as diabetic. Rats found with permanent NIDDM were used for the antidiabetic study.

This model has been used in earlier studies to induce type II diabetes in rats (Neeli, *et al.*, 2007; Venkatesh, *et al.*, 2008). Glipizide dose level at 5 mg/kg was used as the standard drug (Chitra, *et al.*, 2010) .

3.9.4 Experimental design

In this experiment, 42 rats which are 36 rats as diabetic surviving rats and 6 rats as normal rats were divided into seven groups of six rats each group have been used. Diabetic rats were designated as untreated diabetic (normal control), diabetic treated with *L. flavescens* extract doses level at 0.2 and 0.5 g/kg, body weight and the diabetic treated with Glipizide dose level at 5 mg/kg, body weight. Hypoglycemic potential of the compound was studied estimating the blood glucose levels during treatment days within 20 days (Annie, *et al.*, 2005).

Group I: Normal control rats orally administered with distilled water,

Group II: Diabetic control rats orally administered with distilled water,

Group III: Diabetic rats orally administered with Glipizide dose level at 5 mg/kg,

Group IV: Diabetic rats orally administered with Leaves Water Extract dose level at 0.2 g/kg,

Group V: Diabetic rats orally administered with Leaves Water Extract dose level at 0.5 g/kg,

Group VI: Diabetic rats orally administered with Stems Water Extract dose level at 0.2 g/kg,

Group VII: Diabetic rats orally administered with Stems Water Extract dose level at 0.5 g/kg.

Each experimental animal group was orally administered daily with a single dose water extract of *L. flavescens* and Glipizide for 20 days (treatment days). The fasting blood

glucose level (FBGL) have been done by collection of blood from a tail vein and fasting body weight (BW) of rats were monitored for every 5 days throughout the experiment on 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day of the experiment. All experiments were carried out in overnight fasted rats. At the end of the experimental period (day 20^{th}), the animals were deprived of food overnight and then sacrificed by cervical decapitation. Blood was collected in tube containing heparin for the estimation of blood glucose level and the plasma was separated for the estimation of serum lipid profile and liver function test was subjected for biochemical estimation.

3.9.5 Measurement of fasting blood glucose level (FBGL)

The effect of *L. flavescens* water extracts on fasting blood glucose levels (FBGL) in diabetic rats were compared to normal control and diabetic control (Table 4.21). The fasting blood glucose level (FBGL) have been done by collection of blood from a tail vein of rats were monitored for every 5 days throughout the experiment on 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day of the experiment. All experiments were carried out in overnight fasted rats. Significant reduction was observed in the extract treated rats.

3.9.6 Biochemical analysis: Estimation of lipid profile and liver function

At the end of the experimental period (day 20^{th}), the animals were deprived of food overnight and then sacrificed by cervical decapitation. Blood was collected in tube containing heparin for the estimation of blood glucose level and the plasma was separated for the estimation of serum lipid profile and liver function test was subjected for biochemical estimation. The effect of *L. flavescens* water extracts on serum lipid profile and liver function were described at Table 4.22 & Table 4.23 respectively. A decrease in the serum triglycerides, total cholesterol, low density lipids (LDL) and an increase in the high density lipids (HDL), cholesterol levels were observed. The alanine

aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total protein also were observed as effect of *L. flavescens* extract on liver function in diabetic rats.

3.9.7 Measurement of changes in body weight (BW)

Fasting body weight (BW) of rats were monitored for every 5 days throughout the experiment on 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day of the experiment. All experiments were carried out in overnight fasted rats. Figure 4.25 depicts that the effect of *L. flavescens* water extracts on body weight of diabetic rats.

3.10 STATISTICAL ANALYSIS

Data were statistically evaluated using one-way ANOVA for biochemical analysis to estimation of lipid profile and liver function, followed by Turkey and Games-Howell test using SPSS 14.0 software. The values were considered statistically significant when $p<0.05$ level. While the measurement of FBGL and changes in body weight were statistically evaluated using two-way ANOVA, followed by Turkey and Games-Howell test using SPSS 14.0 software. The values were considered statistically significant when $p<0.05$ level. All the results were expressed as mean ± S.E.

CHAPTER 4

4.1 EXTRACTION OF *LEPTOSPERMUM FLAVESCENS* CHEMICAL COMPOUNDS

In this study two parts of *Leptospermum flavescens*; leaves and stems were selected. Hexane, chloroform and water were used to extract the leaves and stems of *L. flavescens* separately. Extraction process was started with low polarity: hexane, followed by medium polarity, chloroform and finally with high polarity, water. The polar solvent extracted out the polar compound and the non-polar compound extracted by the non-polar solvent. 150 ml of solvent was added to 20 g of dried herbs samples in a conical flask. For hexane and chloroform extraction, the samples of leaves and stems were extracted by using water bath at 40 ^{o}C for 24 hours. While the samples of leaves and stems that were extracted with water by using water bath at 100 °C for 24 hours.

The most important factor that influenced the solubility of material was the polarity of the solvent and the solute molecules. The solubility of solid in liquid was indicated by the affinity of molecules of the solvent. Non-polar solvent such as hexane and chloroform were used and the only force presented between molecules was dispersion, because of the production of transient charges induced in the individual molecules. Non polar solvents consisting of disordered molecules allowed the introduction of other non polar molecules easily. In this experiment, the hexane dissolved the non polar compounds such as fats and waxes, while polar solvents such as water dissolved the polar compounds such as alkaloid and sugar.

4.2 SEPARATION OF CHEMICAL COMPOUNDS

A) Thin Layer Chromatography (TLC)

i. Thin Layer Chromatography of hexane crude extract from the leaves of *L. flavescens* by using 10% methanol in chloroform solvent system.

The leaves of *L. flavescens* in the powder form were extracted with hexane solvent and leaves extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using methanol: chloroform (10: 90, v/v) as a solvent system. The appearance of the chemical compounds in the leaves extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This leaves of hexane crude extract, 19 labeled compounds were separated; LHA1, LHA2, LHA3, LHA4, LHA5, LHA6, LHA7, LHA8, LHA9, LHA10, LHA11, LHA12, LHA13, LHA14, LHA15, LHA16, LHA17, LHA18 and LHA19. Table 4.1 showed 9 labeled compounds were identified as flavonoid, 1 labeled compound was identified as saponin and 1 labeled compound was identified as alkaloid.

Table 4.1 Thin Layer Chromatography of hexane crude extract from the leaves of *L. flavescens* by using 10% methanol in chloroform solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Leaves Hexane	LHA 1	11.4	-	Grey (+)	-	Grey (+++)	-	-
	LHA 2	20.0	-	Grey (+)	-	Grey (+++)	-	-
	LHA 3	21.4	-	Grey (+)	-	Blue (+++)	-	Flavonoid
	LHA 4	24.3	-	Grey (+)	-	Grey (+)	-	-
	LHA 5	31.4	-	Grey (+)	-	Blue (+++)	-	Saponin
	LHA 6	32.9	-	Grey (+)	-	Grey (+)	-	-
	LHA 7	41.4	-	Grey (+)	-	Blue (+)	-	-
	LHA 8	42.9	-	Grey (+)	-	Blue (+++)	-	Flavonoid
	LHA 9	47.1	-	Grey (+)	-	Blue (+)	-	-
	LHA 10	48.6	-	Grey (+)	-	Blue (+)	-	-
	LHA 11	52.9	-	Grey (+)	-	Grey (+)	-	-
	LHA 12	54.3	Green (+++)	Grey (+)	Green (+++)	Green (+++)	Yellow (+)	Flavonoid
	LHA 13	58.6	-	Grey (+)	-	Grey (+)	-	-
	LHA 14	62.9	-	Grey (+)	-	Grey (+++)	-	Flavonoid
	LHA 15	67.1	Yellow (+)	Yellow (++)	-	Grey (+++)	-	Flavonoid
	LHA 16	68.6	-	Grey (+)	-	Grey (+++)	-	Flavonoid
	LHA 17	85.7	Green (+)	Yellow (++)	Orange (+)	Grey (+++)	Purple (+)	Alkaloid & Flavonoid
	LHA 18	95.7	Green (+)	Yellow (++)	Yellow (+)	Green (+++)	Purple (+++)	Flavonoid
	LHA 19	98.6	Yellow (+++)	Yellow (++)	Yellow (+++)	Blue (+++)	Purple (+)	Flavonoid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

ii. Thin Layer Chromatography of crude hexane extract from the leaves of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

The leaves of *L. flavescens* in the powder form were extracted with hexane solvent and leaves extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using toluene: ethyl acetate (93: 7, v/v) as a solvent system. The appearance of the chemical compounds in the leaves extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This leaves of hexane crude extract, 23 labeled compounds were separated; LHC1, LHC2, LHC3, LHC4, LHC5, LHC6, LHC7, LHC8, LHC9, LHC10, LHC11, LHC12, LHC13, LHC14, LHC15, LHC16, LHC17, LHC18, LHC19, LHC20, LHC21, LHC22 and LHC23. Table 4.2 showed 11 labeled compounds were identified as flavonoid, 6 labeled compounds were identified as saponin, 3 labeled compounds were identified as terpenoid and 1 labeled compound was identified as alkaloid.

Table 4.2 Thin Layer Chromatography of hexane crude extract from the leaves of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Leaves Hexane	LHC 1	5.7	Yellow (+++)	Grey (+)	-	Blue (+++)	Purple (+)	Flavonoid
	LHC 2	8.6	-	Grey (+)	-	Blue (+++)	-	-
	LHC 3	14.3	-	Grey (+)	-	Grey (+++)	-	-
	LHC 4	18.6	-	Grey (+)	-	Grey (+++)	-	-
	LHC 5	20.0	Yellow (+)	Grey (+)	Green (+)	Grey (+++)	Green (+)	Flavonoid
	LHC 6	22.9	-	Grey (+)	-	Grey (+++)	-	-
	LHC 7	30.0	-	-	-	Blue (+++)	-	-
	LHC 8	31.4	Green (+)	Grey (+)	Green (+)	Blue (+++)	Black (+)	Saponin
	LHC 9	32.9	-	-	-	Blue (+++)	-	-
	LHC 10	35.7	-	-	-	Blue (+++)	Black (+)	Saponin
	LHC 11	38.6	Green (+)	Grey (+)	Green (+++)	Blue (+++)	Green (+++)	Flavonoid
	LHC 12	41.4	-	-	-	Blue (+++)	Yellow (+)	Flavonoid
	LHC 13	44.3	Green (+++)	Grey (+)	Green (+++)	Green (+++)	Black (+++)	Saponin
	LHC 14	47.1	Green (+)	Grey (+)	Yellow (++)	Green (+++)	Purple (+)	Flavonoid
	LHC 15	52.9	-	-	-	Purple (+)	Purple (+++)	Terpenoid & Flavonoid
	LHC 16	57.1	Yellow (+)	Grey (+)	Yellow (+)	Blue (+++)	Black (+)	Saponin
	LHC 17	58.6	-	Grey (+)	-	Grey (+)	Black (+)	Saponin
	LHC 18	68.6	-	Grey (+)	-	Purple (+)	Black (+)	Terpenoid & Saponin
	LHC 19	72.9	-	Grey (+)	-	Grey (+)	Orange (+)	Flavonoid
	LHC 20	78.6	-	Grey (+)	-	Blue (+)	Red (+)	Flavonoid
	LHC 21	82.9	-	Yellow (++)	-	Orange (+)	Yellow (+)	Flavonoid
	LHC 22	90.0	-	-	Orange (++)	Purple (+)	Yellow (+)	Alkaloid , Terpenoid & Flavonoid
	LHC 23	98.6	Yellow (+)	-	Yellow (+++)	Blue (+++)	Purple (+)	Flavonoid

iii. Thin Layer Chromatography of chloroform crude extract from the leaves of *L. flavescens* by using 10 % methanol in chloroform solvent system.

The leaves of *L. flavescens* in the powder form were extracted with chloroform solvent and leaves extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using methanol: chloroform (10: 90, v/v) as a solvent system. The appearance of the chemical compounds in the leaves extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This leaves of chloroform crude extract, 21 labeled compounds were separated; LCA1, LCA2, LCA3, LCA4, LCA5, LCA6, LCA7, LCA8, LCA9, LCA10, LCA11, LCA12, LCA13, LCA14, LCA15, LCA16, LCA17, LCA18, LCA19, LCA20, and LCA21. Table 4.3 showed 8 labeled compounds were identified as flavonoid, 2 labeled compounds were identified as terpenoid, and 1 labeled compound was identified as alkaloid.

Table 4.3 Thin Layer Chromatography of chloroform crude extract from the leaves of *L. flavescens* by using 10 % methanol in chloroform solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Leaves Chloroform	LCA 1	2.9	Green (+)	-	-	Grey (+++)	-	-
	LCA 2	4.3	Green (+)	-	-	Grey (+)	-	-
	LCA 3	7.1	Green (+++)	Grey (+)	-	Green (+++)	-	-
	LCA 4	12.9	-	-	-	Blue (+)	-	-
	LCA 5	17.1	Green (+)		-	Green (+++)	Purple (+)	Flavonoid
	LCA 6	22.9	-	-	-	Blue (+)	-	-
	LCA 7	32.9	-	-	-	Blue (+++)	-	-
	LCA 8	51.4	-	-	-	Blue (+++)	-	-
	LCA 9	55.7	Green (+)	Grey (+)	Green (+++)	Blue (+++)	Green (+)	Flavonoid
	LCA 10	61.4	-	-	-	Purple (+++)	-	Terpenoid
	LCA 11	68.6	-	-	-	Purple (+)	-	Terpenoid
	LCA 12	72.9	-	-	-	Green (+)	-	-
	LCA 13	75.7	-	-	-	Yellow (+)	-	-
	LCA 14	78.6	-	-	-	Green (+)	-	-
	LCA 15	80.0	-	-	-	Blue (+++)	-	-
	LCA 16	82.9	Green (++)	Grey (+)	Green (+)	Yellow (+)	Green (+++)	Flavonoid
	LCA 17	87.1	Yellow (+++)	Grey (+)	Green (+)	Blue (+++)	Yellow (+++)	Flavonoid
	LCA 18	87.1	Green (+)	Grey (+)	Yellow (+)	Green (+)	Green (+)	Flavonoid
	LCA 19	92.9	Green (+++)	Grey (+)	Green (+)	Green (+++)	Yellow (+)	Flavonoid
	LCA 20	95.7	Yellow (+)	Grey (+)	Orange (++)	Blue (+)	Purple (+)	Alkaloid & Flavonoid
	LCA 21	98.6	Yellow (+)	Grey (+)	Green (+++)	Blue (+++)	Green (+)	Flavonoid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

iv. Thin Layer Chromatography of chloroform crude extract from the leaves of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

The leaves of *L. flavescens* in the powder form were extracted with chloroform solvent and leaves extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using toluene: ethyl acetate (93: 7, v/v), as a solvent system. The appearance of the chemical compounds in the leaves extract were observed under visible light, UV light and then spray by reagents including Dragendorff, Vanilin and Anisaldehyde. This leaves of chloroform crude extract, 16 labeled compounds were separated; LCC1, LCC2, LCC3, LCC4, LCC5, LCC6, LCC7, LCC8, LCC9, LCC10, LCC11, LCC12, LCC13, LCC14, LCC15 and LCC16. Table 4.4 showed 4 labeled compounds were identified as flavonoid, 2 labeled compounds were identified as saponin, 2 labeled compounds were identified as alkaloid and 1 labeled compound was identified as terpenoid.

Table 4.4 Thin Layer Chromatography of chloroform crude extract from the leaves of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Leaves Chloroform	LCC 1	4.3	Yellow (+)	Yellow (+)	Orange (+)	Blue (+++)	-	Alkaloid
	LCC 2	5.7	-	-	-	Blue (+++)	-	-
	LCC 3	11.4	-	-	-	Blue (+++)	-	-
	LCC 4	21.4	Green (+++)	Grey (+)	Green (+)	Green (+++)	Red (+)	Flavonoid
	LCC 5	30.0	-	-	-	Green (+)	-	-
	LCC 6	31.4	Green (+++)	Grey (+)	Green (+)	Green (+)	Black (+)	Saponin
	LCC 7	34.3	Green (+++)	Green (+)	Green (+++)	Green (+)	Green (+)	Flavonoid
	LCC 8	37.1	Green (+++)	Grey (+)	Green (+++)	Green (++)	Green (+++)	Flavonoid
	LCC 9	40.0	Green (+++)	Grey (+)	Green (+)	Green (+++)	Black (+)	Saponin
	LCC 10	41.4	Green (+++)	Grey (++)	Green (+++)	Green (+++)	Yellow (+)	Flavonoid
	LCC 11	45.7	-	-	-	Grey (+)	-	-
	LCC 12	51.4	-	-	-	Green (+++)	-	-
	LCC 13	74.3	-	-	-	Blue (+)	-	-
	LCC 14	77.1	-	-	-	Grey (+)	-	-
	LCC 15	88.6	-	-	-	Purple (+)	-	Terpenoid
	LCC 16	97.1	-	-	Orange (+)	Blue (+)	-	Alkaloid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

v. Thin Layer Chromatography of water crude extract from the leaves of *L. flavescens* by using 10 % methanol in chloroform solvent system.

The leaves of *L. flavescens* in the powder form were extracted with water and leaves extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using methanol: chloroform (10: 90, v/v) as a solvent system. The appearance of the chemical compounds in the leaves extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This leaves of water crude extract, 20 labeled compounds were separated; LDA1, LDA2, LDA3, LDA4, LDA5, LDA6, LDA7, LDA8, LDA9, LDA10, LDA11, LDA12, LDA13, LDA14, LDA15, LDA16, LDA17, LDA18, LDA19, LDA20. Table 4.5 showed 11 labeled compounds were identified as flavonoid, 5 labeled compounds were identified as saponin, 3 labeled compounds were identified as terpenoid and 1 labeled compound was identified as alkaloid.

Table 4.5 Thin Layer Chromatography of water crude extract from the leaves of *L. flavescens* by using 10 % methanol in chloroform solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Leaves Water	LDA 1	5.7	Yellow (+++)	Grey (+)	-	Blue (+++)	Purple (+)	Flavonoid
	LDA 2	8.6	Yellow (+)	Grey (+)	Green (+)	Grey (+++)	Green (+)	Flavonoid
	LDA 3	14.3	-	Grey (+)	-	Grey (+++)	-	-
	LDA 4	18.6	-	-	-	Blue (+++)	-	-
	LDA 5	20.0	Green (+)	Grey (+)	Green (+)	Blue (+++)	Black (+)	Saponin
	LDA 6	22.9	-	-	-	Blue (+++)	-	-
	LDA 7	30.0	-	-	-	Blue (+++)	Black (+)	Saponin
	LDA 8	31.4	Green (+)	Grey (+)	Green (+++)	Blue (+++)	Green (+++)	Flavonoid
	LDA 9	32.9	-	-	-	Blue (+++)	Yellow (+)	Flavonoid
	LDA 10	35.7	Green (+++)	Grey (+)	Green (+++)	Green (+++)	Black (+++)	Saponin
	LDA 11	38.6	Green (+)	Grey (+)	Yellow (++)	Green (+++)	Purple (+)	Flavonoid
	LDA 12	41.4	-	-	-	Purple (+)	Purple (+++)	Terpenoid & Flavonoid
	LDA 13	44.3	Yellow (+)	Grey (+)	Yellow (+)	Blue (+++)	Black (+)	Saponin
	LDA 14	47.1	-	Grey (+)	-	Grey (+)	Black (+)	Saponin
	LDA 15	52.9	-	Grey (+)	-	Purple (+)	Black (+)	Terpenoid & Saponin
	LDA 16	57.1	-	Grey (+)	-	Grey (+)	Orange (+)	Flavonoid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

vi. Thin Layer Chromatography of water crude extract from the leaves of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

The leaves of *L. flavescens* in the powder form were extracted with water and leaves extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using toluene: ethyl acetate (93: 7, v/v) as a solvent system. The appearance of the chemical compounds in the leaves extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This leaves of water crude extract, 16 labeled compounds were separated; LDC1, LDC2, LDC3, LDC4, LDC5, LDC6, LDC7, LDC8, LDC9, LDC10, LDC11, LDC12, LDC13, LDC14, LDC15 and LDC16. Table 4.6 showed 9 labeled compounds were identified as flavonoid, 1 labeled compound was identified as saponin and 1 labeled compound was identified as alkaloid.

Table 4.6 Thin Layer Chromatography of water crude extract from the leaves of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Leaves Water	LDC 1	21.4	-	Grey (+)	-	Blue (+++)	-	Flavonoid
	LDC 2	31.4	-	Grey (+)	-	Blue (+++)	-	Saponin
	LDC 3	32.9	-	Grey (+)	-	Grey (+)	-	-
	LDC 4	41.4	-	Grey (+)	-	Blue (+)	-	-
	LDC 5	42.9	-	Grey (+)	-	Blue (+++)	-	Flavonoid
	LDC 6	47.1	-	Grey (+)	-	Blue (+)	-	-
	LDC 7	48.6	-	Grey (+)	-	Blue (+)	-	-
	LDC 8	52.9	-	Grey (+)	-	Grey (+)	-	-
	LDC 9	54.3	Green (+++)	Grey (+)	Green (+++)	Green (+++)	Yellow (+)	Flavonoid
	LDC 10	58.6	-	Grey (+)	-	Grey (+)	-	-
	LDC 11	62.9	-	Grey (+)	-	Grey (+++)	-	Flavonoid
	LDC 12	67.1	Yellow (+)	Yellow (++)	-	Grey (+++)	-	Flavonoid
	LDC 13	68.6	-	Grey (+)	-	Grey (+++)	-	Flavonoid
	LDC 14	85.7	Green (+)	Yellow (++)	Orange (+)	Grey (+++)	Purple (+)	Alkaloid & Flavonoid
	LDC 15	95.7	Green (+)	Yellow (++)	Yellow (+)	Green (+++)	Purple (+++)	Flavonoid
	LDC 16	98.6	Yellow (+++)	Yellow (++)	Yellow (+++)	Blue (+++)	Purple (+)	Flavonoid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

vii. Thin Layer Chromatography of hexane crude extract from the stems of *L. flavescens* by using 10 % methanol in chloroform solvent system.

The stems of *L. flavescens* in the powder form were extracted with hexane solvent and stems extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using methanol: chloroform (10: 90, v/v) as a solvent system. The appearance of the chemical compounds in the stems extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This stems of hexane crude extract, 15 labeled compounds were separated; SHA1, SHA2, SHA3, SHA4, SHA5, SHA6, SHA7, SHA8, SHA9, SHA10, SHA11, SHA12, SHA13, SHA14 and SHA15. Table 4.7 showed 4 labeled compounds were identified as flavonoid, 2 labeled compounds were identified as terpenoid, 2 labeled compounds were identified as essential oil and 1 labeled compound was identified as saponin.

Table 4.7 Thin Layer Chromatography of hexane crude extract from the stems of *L. flavescens* by using 10 % methanol in chloroform solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Stems Hexane	SHA 1	5.7	-	Grey (+)	-	Grey (+++)	-	-
	SHA 2	17.1	-	Grey (+)	-	Grey (+++)	-	-
	SHA 3	31.4	-	Grey (+)	-	Blue (+)	-	-
	SHA 4	52.9	-	Grey (+)	-	Grey (+)	-	-
	SHA 5	58.6	-	Grey (+)	-	Grey (+)	-	-
	SHA 6	70.0	-	Grey (+)	-	Grey (+)	-	-
	SHA 7	80.0	-	Grey (+)	-	Purple (+)	-	Terpenoid
	SHA 8	82.9	-	Grey (+)	-	Blue (+)	Red (+)	Flavonoid
	SHA 9	84.3	Yellow (+)	Grey (+)	-	Purple (+)	Blue (+)	Terpenoid & Essential oil
	SHA 10	87.1	-	Grey (+)	-	Blue (+)	Black (+)	Saponin
	SHA 11	90.0	-	Grey (+)	-	Blue (+++)	Blue (+)	Essential oil
	SHA 12	91.4	-	Grey (+)	-	Green (+)	Yellow (+)	Flavonoid
	SHA 13	94.3	Green (+)	Grey (+)	-	Green (+)	-	-
	SHA 14	97.1	Green (+++)	Grey (+)	-	Green (+++)	Green (+)	Flavonoid
	SHA 15	98.6	Yellow (+++)	Yellow (++)	Green (+)	Blue (+++)	Yellow (+)	Flavonoid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

viii. Thin Layer Chromatography of hexane crude extract from the stems of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

The stems of *L. flavescens* in the powder form were extracted with hexane solvent and stems extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using toluene: ethyl acetate (93: 7, v/v) as a solvent system. The appearance of the chemical compounds in the stems extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This stems of hexane crude extract, 19 labeled compounds were separated; SHC1, SHC2, SHC3, SHC4, SHC5, SHC6, SHC7, SHC8, SHC9, SHC10, SHC11, SHC12, SHC13, SHC14, SHC15, SHC16, SHC17, SHC18 and SHC19. Table 4.8 showed 5 labeled compounds were identified as terpenoid, 2 labeled compounds were identified as saponin, 1 labeled compound was identified as flavonoid and 1 labeled compound was identified as alkaloid.

Table 4.8 Thin Layer Chromatography of hexane crude extract from the stems of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Stems Hexane	SHC 1	2.9	Yellow (+)	Grey (+)	-	Grey (+++)	-	-
	SHC 2	11.4	-	Grey (+)	-	Blue (+++)	-	-
	SHC 3	17.1	-	Grey (+)	-	Blue (+++)	-	-
	SHC 4	21.4	Green (+)	Grey (+)	Green (+)	Blue (+++)	-	-
	SHC 5	24.3	-	Grey (+)	-	Blue (+++)	-	-
	SHC 6	28.6	-	Grey (+)	-	Blue (+++)	-	-
	SHC 7	31.4	Green (+)	-	Green (+)	Blue (+++)	Black (+)	Saponin
	SHC 8	34.3	-	Grey (+)	-	Blue (+++)	-	-
	SHC 9	37.1	Green (+++)	-	Green (+)	Blue (+++)	Black (+)	Saponin
	SHC 10	40.0	Green (+)	-	Green (+)	Blue (+++)	Green (+)	Flavonoid
	SHC 11	42.9	-	-	-	Grey (+)	-	-
	SHC 12	45.7	-	-	-	Purple (+)	-	Terpenoid
	SHC 13	51.4	-	-	-	Purple (++)	-	Terpenoid
	SHC 14	57.1	-	-	-	Blue (+)	-	-
	SHC 15	61.4	-	-	-	Purple (+)	-	Terpenoid
	SHC 16	67.1	-	-	-	Purple (+)	-	Terpenoid
	SHC 17	74.3	-	-	-	Blue (+)	-	-
	SHC 18	85.7	-	-	-	Purple (++)	-	Terpenoid
	SHC 19	97.1	-	-	Orange	Grey (+)	-	Alkaloid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

ix. Thin Layer Chromatography of chloroform crude extract from the stems of *L. flavescens* by using 10 % methanol in chloroform solvent system.

The stems of *L. flavescens* in the powder form were extracted with chloroform solvent and stems extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using methanol: chloroform (10: 90, v/v) as a solvent system. The appearance of the chemical compounds in the stems extract were observed under visible light, UV light and then by spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This stems of chloroform crude extract, 15 labeled compounds were separated; SCA1, SCA2, SCA3, SCA4, SCA5, SCA6, SCA7, SCA8, SCA9, SCA10, SCA11, SCA12, SCA13, SCA14 and SCA15. Table 4.9 showed 2 labeled compounds were identified as flavonoid and 1 labeled compound was identified as essential oil.

Table 4.9 Thin Layer Chromatography of chloroform crude extract from the stems of *L. flavescens* by using 10 % methanol in chloroform solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Stems Chloroform	SCA 1	4.3	Green (+)	-	-	Grey (+++)	-	-
	SCA 2	7.1	Green (+)	-	-	Green (+++)	-	-
	SCA 3	11.4	-	-	-	Blue (+)	-	-
	SCA 4	22.9	-	-	-	Blue (+++)	-	-
	SCA 5	41.4	-	-	-	Blue (+++)	-	-
	SCA 6	45.7	-	-	-	Blue (+++)	-	-
	SCA 7	54.3	-	-	-	Blue (+)	-	-
	SCA 8	61.4	-	-	-	Blue (+++)	-	-
	SCA 9	67.1	-	-	-	Green (+)	-	-
	SCA 10	71.4	-	-	-	Blue (+)	-	-
	SCA 11	75.7	Green (+)	Grey (+)	Green (+)	Green (+)	-	-
	SCA 12	82.9	Green (+)	Grey (+)	Green (+)	Green (+)	Green (+)	Flavonoid
	SCA 13	92.9	-	-	-	Blue (+)	-	-
	SCA 14	95.7	Yellow (+)	-	-	Blue (+++)	Blue (+)	Essential oil
	SCA 15	98.6	Green (+++)	Grey (+)	Green (+++)	Green (+++)	Green (+)	Flavonoid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

x. Thin Layer Chromatography of chloroform crude extract from the stems of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

The stems of *L. flavescens* in the powder form were extracted with chloroform solvent and stems extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using toluene: ethyl acetate (93: 7, v/v) as a solvent system. The appearance of the chemical compounds in the stems extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. In this stems of chloroform extract, 12 labeled compounds were separated; SCC1, SCC2, SCC3, SCC4, SCC5, SCC6, SCC7, SCC8, SCC9, SCC10, SCC11 and SCC12. Table 4.10 showed 2 labeled compounds were identified as flavonoid, 1 labeled compound was identified as alkaloid and 1 labeled compound was identified as terpenoid.

Table 4.10 Thin Layer Chromatography of chloroform crude extract from the stems of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Stems Chloroform	SCC 1	4.3	Yellow (+)	Yellow (+)	-	Blue (+++)	-	-
	SCC 2	11.4	-	-	-	Blue (+++)	-	-
	SCC 3	17.1	-	-	-	Blue (+)	-	-
	SCC 4	21.4	Green (+)	Grey (+)	Orange (+)	Blue (+)	-	Alkaloid
	SCC 5	24.3	-	-	-	Blue (+)	-	-
	SCC 6	30.0	Green (+++)	Grey (+)	Green (+)	Green (+++)	Green (+)	Flavonoid
	SCC 7	31.4	-	-	-	Blue (+)	-	-
	SCC 8	35.7	Green (+++)	Grey (+)	Green (+++)	Green (++)	Green (+)	Flavonoid
	SCC 9	38.6	-	-	-	Green (+)	-	-
	SCC 10	48.6	-	-	-	Purple (+)	-	Terpenoid
	SCC 11	87.1	-	-	-	Blue (+)	-	-
	SCC 12	98.6	-	-	-	Blue (+)	-	-

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

xi. Thin Layer Chromatography of water crude extract from the stems of *L. flavescens* by using 10 % methanol in chloroform solvent system.

The stems of *L. flavescens* in the powder form were extracted with water and stems extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using methanol: chloroform (10: 90, v/v) as a solvent system. The appearance of the chemical compounds in the stems extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This stems of water extract, 14 labeled compounds were separated; SDA1, SDA2, SDA3, SDA4, SDA5, SDA6, SDA7, SDA8, SDA9, SDA10, SDA11, SDA12, SDA13 and SDA14. Table 4.11 showed 8 labeled compounds were identified as flavonoid, 3 labeled compounds were identified as terpenoid, 2 labeled compounds were identified as essential oil, 1 labeled compound was identified as alkaloid and 1 labeled compound was identified as saponin.

Table 4.11 Thin Layer Chromatography of water crude extract from the stems of *L. flavescens* by using 10 % methanol in chloroform solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Stems Water	SDA 1	57.1	-	Grey (+)	-	Grey (+)	Orange (+)	Flavonoid
	SDA 2	58.6	-	Grey (+)	-	Blue (+)	Red (+)	Flavonoid
	SDA 3	68.6	-	Yellow (++)	-	Orange (+)	Yellow (+)	Flavonoid
	SDA 4	72.9	-	-	Orange (++)	Purple (+)	-	Alkaloid & Terpenoid
	SDA 5	78.6	Yellow (+)	-	Yellow (+++)	Blue (+++)	Purple (+)	Flavonoid
	SDA 6	80.0	-	Grey (+)	-	Purple (+)	-	Terpenoid
	SDA 7	82.9	-	Grey (+)	-	Blue (+)	Red (+)	Flavonoid
	SDA 8	84.3	Yellow (+)	Grey (+)	-	Purple (+)	Blue (+)	Terpenoid & Essential oil
	SDA 9	87.1	-	Grey (+)	-	Blue (+)	Black (+)	Saponin
	SDA 10	90.0	-	Grey (+)	-	Blue (+++)	Blue (+)	Essential oil
	SDA 11	91.4	-	Grey (+)	-	Green (+)	Yellow (+)	Flavonoid
	SDA 12	94.3	Green (+)	Grey (+)	-	Green (+)	-	-
	SDA 13	97.1	Green (+++)	Grey (+)	-	Green (+++)	Green (+)	Flavonoid
	SDA 14	98.6	Yellow (+++)	Yellow (++)	Green (+)	Blue (+++)	Yellow (+)	Flavonoid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

xii. Thin Layer Chromatography of water crude extract from the stems of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

The stems of *L. flavescens* in the powder form were extracted with water solvent and stems extract were dried by using rotary evaporator. The aluminium plates were used and TLC was developed by using toluene: ethyl acetate (93: 7, v/v) as a solvent system. The appearance of the chemical compounds in the stems extract were observed under visible light, UV light and then spray by reagents such Dragendorff, Vanilin and Anisaldehyde. This stems of water extract, 12 labeled compounds were separated; SDC1, SDC2, SDC3, SDC4, SDC5, SDC6, SDC7, SDC8, SDC9, SDC10, SDC11 and SDC12. Table 4.12 showed 5 labeled compounds were identified as terpenoid, 1 labeled compound was identified as flavonoid, 1 labeled compound was identified as saponin and 1 labeled compound was identified as alkaloid.

Table 4.12 Thin Layer Chromatography of water crude extract from the stems of *L. flavescens* by using 93 % toluene in ethyl acetate solvent system.

Sample	Label compound	R_f value (X 100)	Observation					Comment
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Anisaldehyde	
Stems Water	SDC 1	21.4	-	Grey (+)	-	Blue (+++)	-	-
	SDC 2	31.4	Green (+++)	-	Green (+)	Blue (+++)	Black (+)	Saponin
	SDC 3	32.9	Green (+)	-	Green (+)	Blue (+++)	Green (+)	Flavonoid
	SDC 4	41.4	-	-	-	Grey (+)	-	-
	SDC 5	42.9	-	-	-	Purple (+)	-	Terpenoid
	SDC 6	47.1	-	-	-	Purple (++)	-	Terpenoid
	SDC 7	48.6	-	-	-	Blue (+)	-	-
	SDC 8	52.9	-	-	-	Purple (+)	-	Terpenoid
	SDC 9	54.3	-	-	-	Purple (+)	-	Terpenoid
	SDC 10	58.6	-	-	-	Blue (+)	-	-
	SDC 11	62.9	-	-	-	Purple (++)	-	Terpenoid
	SDC 12	67.1	-	-	Orange	Grey (+)	-	Alkaloid

Indicator:
(+) = Very light in colour, (++) = Light in colour, (+++) = Dark in colour, (++++) = Very dark in colour

B) High performance liquid chromatography (HPLC)

The HPLC method applied was a modification of that reported (Lima, *et al.*, 2007). Isocratic method was used for crude extract samples of water, chloroform and hexane from the leaves and stems of *L. flavescens*. Isocratic method was a constant composition of mobile phase. 10 to 20 µL of crude extract samples were separated within a total time of 60 minutes and their flow rate were 0.8 ml/min. The peaks were detected at 254 nm. The results then were compared with HPLC chromatogram of the leaves and stems extract *L. flavescens* by using different solvent in order to identify the chemical compounds in *L. flavescens* (Lima, *et al.*, 2007). Acetonitrile-water (5:95, v/v) was using as mobile phase. Acetonitrile was prepared in pump A and water was prepared in pump B. HPLC analysis was developed to separate the presence of the chemical compounds in the leaves and stems crude extract of *L. flavescens*.

Leaves chloroform crude extract of *L. flavescens*.

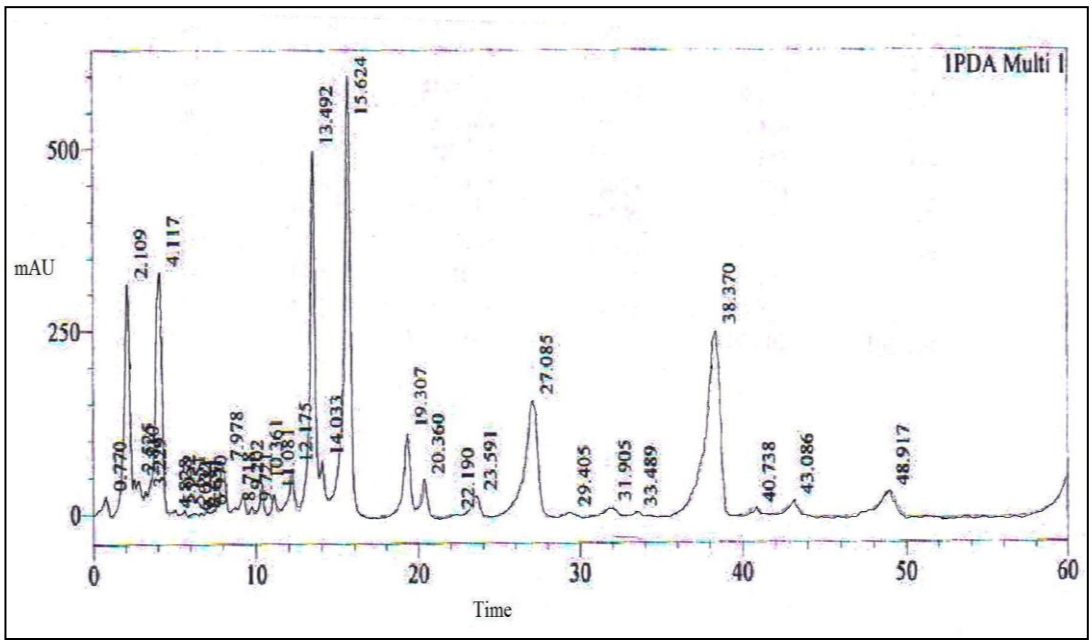

Figure 4.1 HPLC chromatograms of chloroform crude extract from the leaves of *L. flavescens*

From Figure 4.1 it observed that 19 peaks were detected from the leaves chloroform crude extract of *L. flavescens*.

Leaves water crude extract of *L. flavescens*.

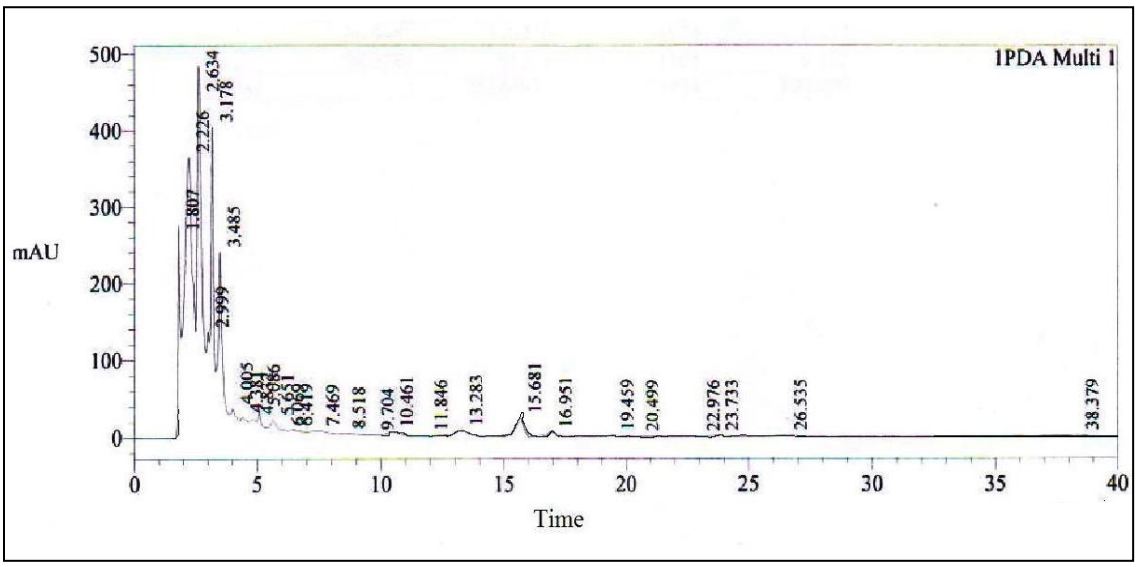

Figure 4.2 HPLC chromatograms of water crude extract from the leaves of *L. flavescens*.

From Figure 4.2 it observed that 10 peaks were detected from the leaves water crude extract of *L. flavescens*.

Stems chloroform crude extract of *L. flavescens*.

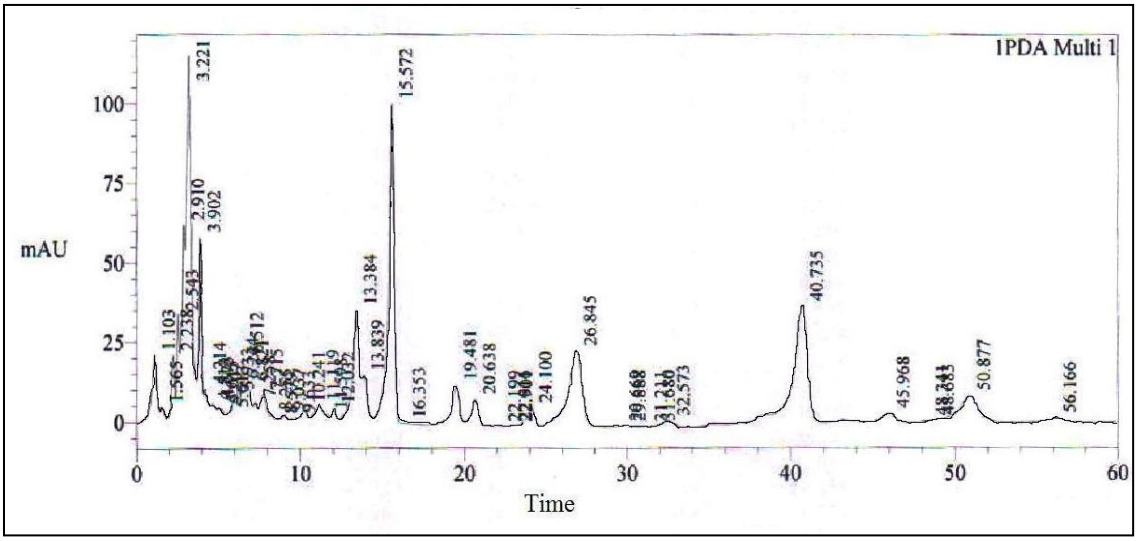

Figure 4.3 HPLC chromatograms of chloroform crude extract from the stems of *L. flavescens*.

From Figure 4.3 it observed that 17 peaks were detected from the stems chloroform crude extract of *L. flavescens*.

Stems water crude extract of *L. flavescens*.

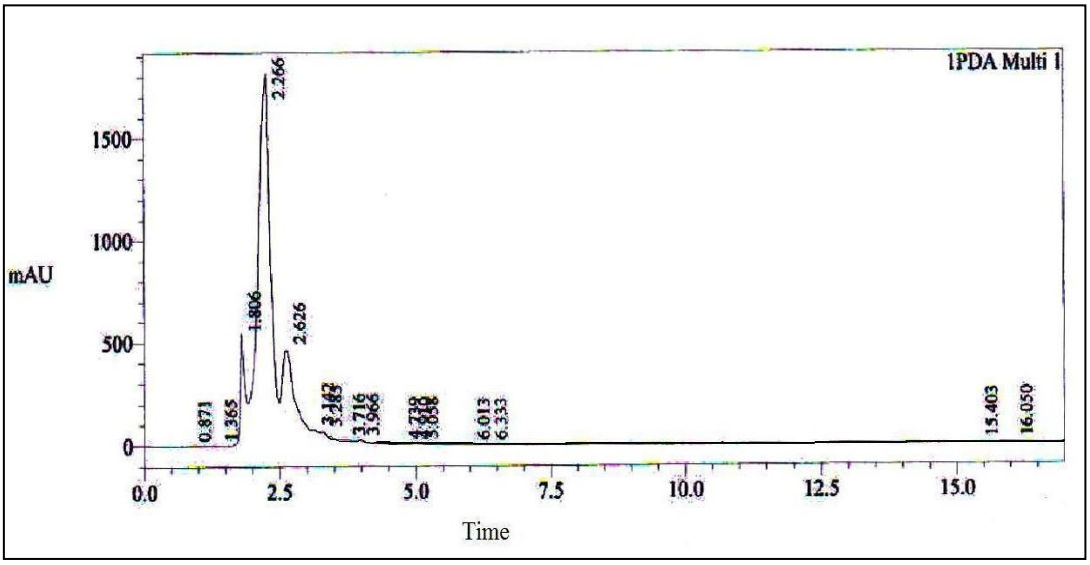

Figure 4.4 HPLC chromatograms of water crude extract from the stems of *L. flavescens*.

From Figure 4.4 it observed that 3 peaks were detected from the stems water crude extract of *L. flavescens*.

i) Standard of quarcetin

10 µL of quarcetin at concentration of 0.01 g/ml was injected into HPLC system with flow rate of 0.8 ml/min. The peaks were detected at 228 nm and identified by standard substances (Lima, *et al.*, 2007). HPLC developed to separated the quarcetin compound. Test sample was separated with two solvent system:

a) 0.05% Trifluoroacetic acid (TFA) in water

b) 0.05% TFA in acetonitrile

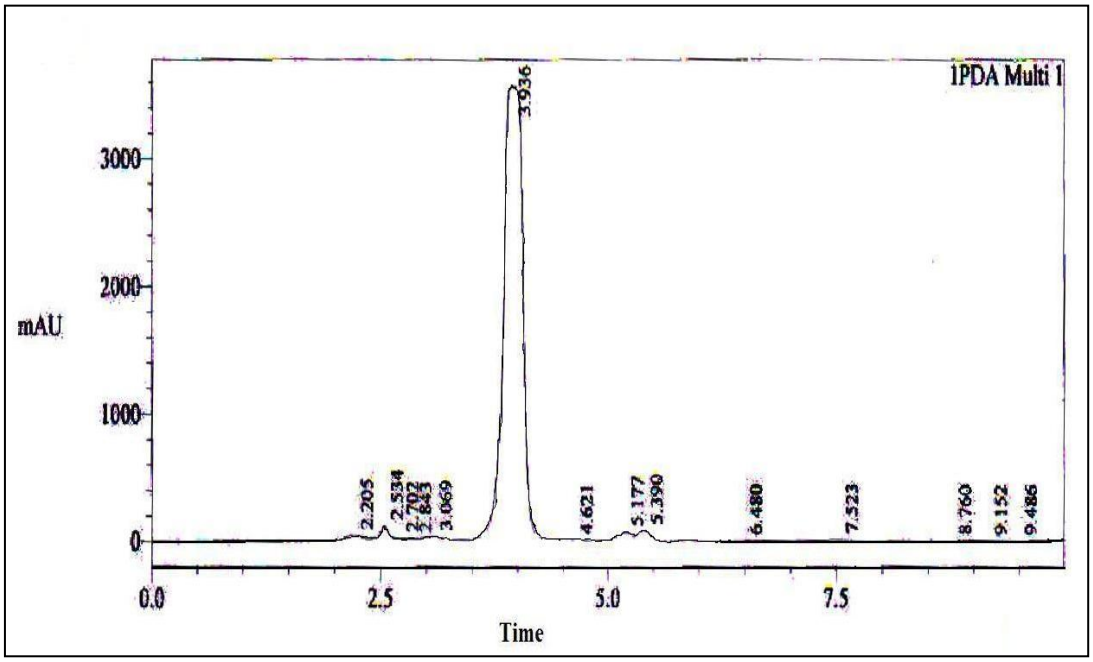

Figure 4.5 HPLC chromatograms of standard quarcetin (0.01 g/ml)

From Figure 4.5 it observed that a standard quarcetin compound showed a highest peak at the concentration of 0.01 g/ml.

ii) Standard of gallic acid

10 µL of gallic acid at concentration of 0.01 g/ml was injected into HPLC system with flow rate of 0.8 ml/min. The peaks were detected at 228 nm and identified by standard substances (Lima, *et al.*, 2007). HPLC developed to separated the gallic acid compound. Test sample was separated with two solvent system:

a) 0.05% Trifluoroacetic acid (TFA) in water

b) 0.05% TFA in acetonitril

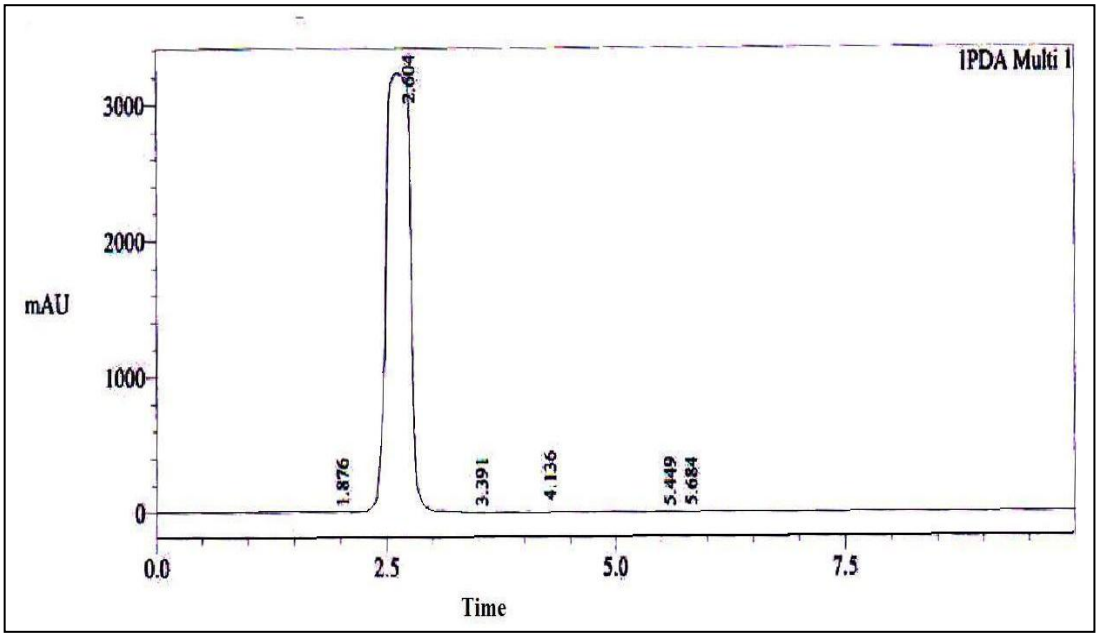

Figure 4.6 HPLC chromatograms of standard gallic acid (0.01 g/ml)

From Figure 4.6 it observed that a standard gallic acid compound showed a highest peak at the concentration of 0.01 g/ml.

iii) Standard of tannic acid

10 µL of tannic acid at concentration of 0.01 g/ml was injected into HPLC system with flow rate of 0.8 ml/min. The peaks were detected at 228 nm and identified by standard substances (Lima, *et al.*, 2007). HPLC developed to separated the tannic acid compound. Test sample was separated with two solvent system:

a) 0.05% Trifluoroacetic acid (TFA) in water

b) 0.05% TFA in acetonitrile

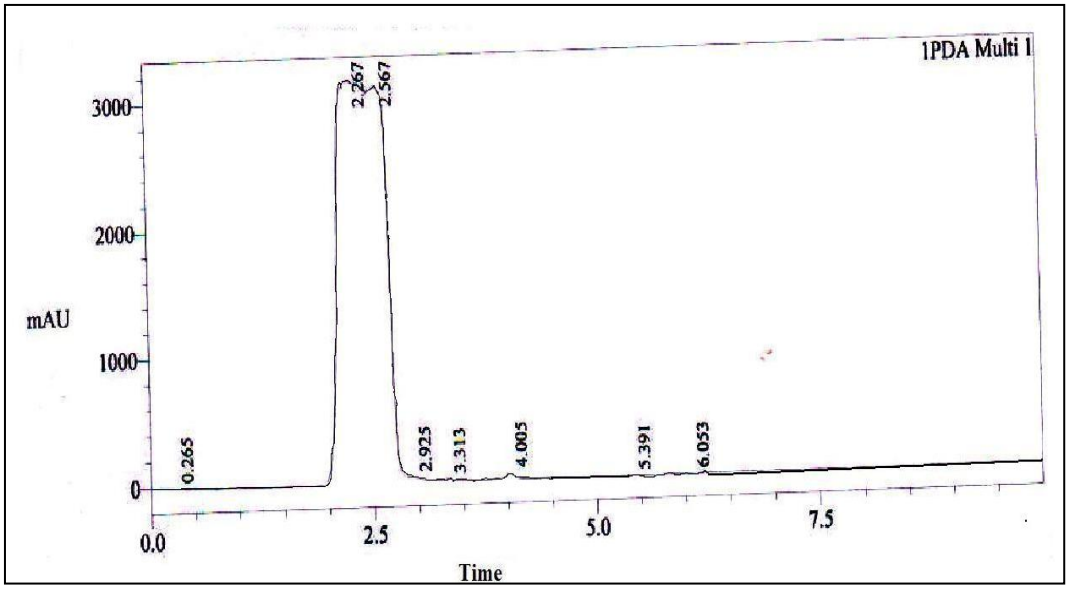

Figure 4.7 HPLC chromatograms of standard tannic acid (0.01 g/ml)

From Figure 4.7 it observed that a standard tannic acid compound showed a highest peak at the concentration of 0.01 g/ml.

C) Liquid chromatography mass spectrometry combined with mass spectrometry (LCMS-MS)

Table 4.13 showed detection compounds of leaves and stems from hexane, chloroform and water crude of *Leptospermum flavescens* by LCMS/MS analyses were performed using full scan with MS/MS data collection. In this study LCMS/MS analyses have been done by Column: Phenomenex Aqua C18-50 mm x 2.0 mm x 5 µM. The buffer A: Water with 0.1 % formic acid and 5 mM ammonium formate. While the buffer B: Acetonitrile with 0.1 % formic acid and 5 mM ammonium formate. Rapid screening at 15 minutes runs time. AB Sciex 3200QTrap LCMS/MS Gradient run programmer: 10 % A to 90 % B from 0.01 minutes to 8.0 minutes hold for 3 minutes and back to 10 % A in 0.1 minutes and re-equilibrated for 4 minutes. Pre-run equilibration time: 1.0 minutes. Electro Spray Ionization (ESI) source was used in negative mode in this study. From the LCMS/MS chromatographic and mass spectrometric data for analyzed compounds, 18 labeled compounds were detected as aromadendrin glucoside, eriodictyol, hydroxybenzyl hexose, hyperin, kaempferol rhamnoside, quercetin or herbacetin rhamnoside, quercetin pentose or narigenin glucoside, quinic acid, vindoline, cinnamic acid, flavonol glycosides, protocatechuic acid, methyl epicatechin gallate, apigenin, 3 methyl epigallocatechin gallate, tetrahydroxy chalcone, hydroxybenzyl hexose- isomers and pinobanksin-5-methyl-ether.

Chromatograms showed the peaks of compounds from hexane, chloroform and water crude of *Leptospermum flavescens*. The full chromatograms of leaves hexane crude extract (Figure 8.1), leaves chloroform crude extract (Figure 8.2), leaves water crude extract (Figure 8.3), stems hexane crude extract (Figure 8.4), stems chloroform crude extract (Figure 8.5) and stems water crude extract (Figure 8.6).

Table 4.13 Compounds detected from crude extract of *Leptospermum flavescens* by LCMS/MS analysis.

Detection of compounds	Extraction of *Leptospermum flavescens*						References
	Leaves hexane	Stems hexane	Leaves chloroform	Stems chloroform	Leaves water	Stems Water	
Aromadendrin glucoside					✓		Figure 4.10
Hydroxybenzyl hexose	✓						Figure 4.9
Hyperin					✓		Figure 4.11
Kaempferol rhamnoside					✓		Figure 4.12
Quercetin or herbacetin rhamnoside					✓		Figure 4.13
Narigenin					✓		Figure 4.14
Quinic Acid			✓		✓	✓	Figure 4.15
Vindoline	✓						Figure 4.8
Flavonol glycosides						✓	Figure 4.16
. Protocatechuic Acid						✓	Figure 4.17
Methyl epicatechin gallate				✓			Figure 4.18
Apigenin			✓				Figure 4.19
Tetrahydroxy chalcone				✓			Figure 4.20

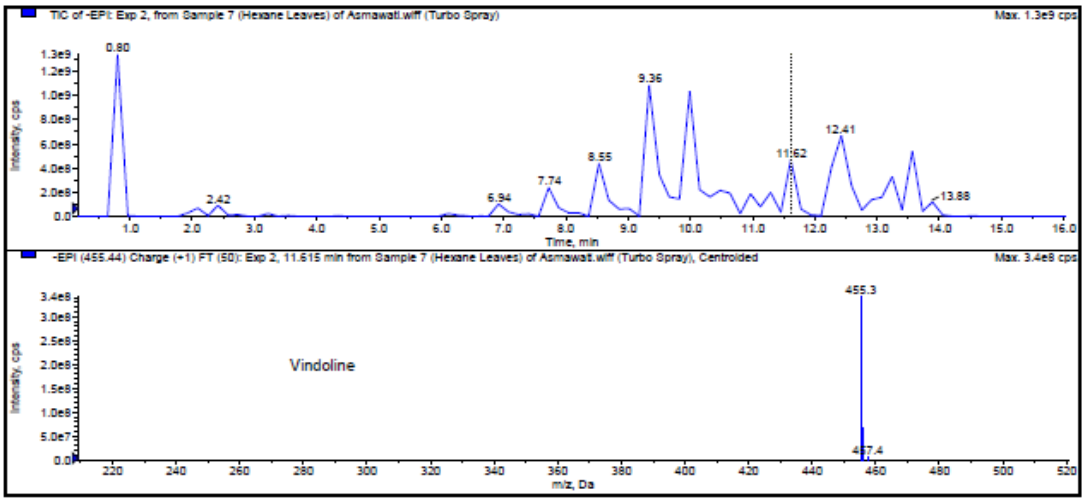

Figure 4.8 LCMS/MS chromatograms of vindoline from leaves hexane crude extract of *L. flavescens*

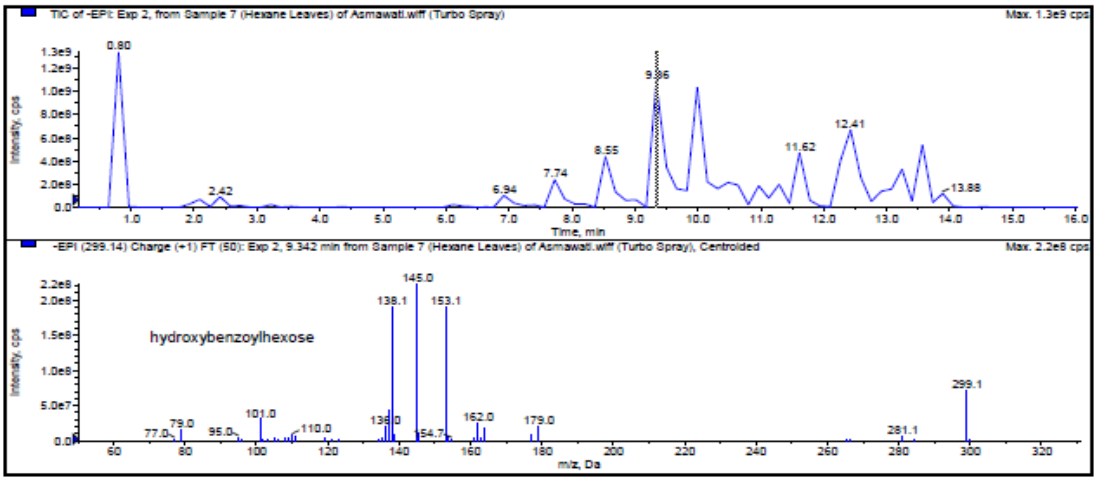

Figure 4.9 LCMS/MS chromatograms of hydroxybenzonylhexose from leaves hexane crude extract of *L. flavescens*

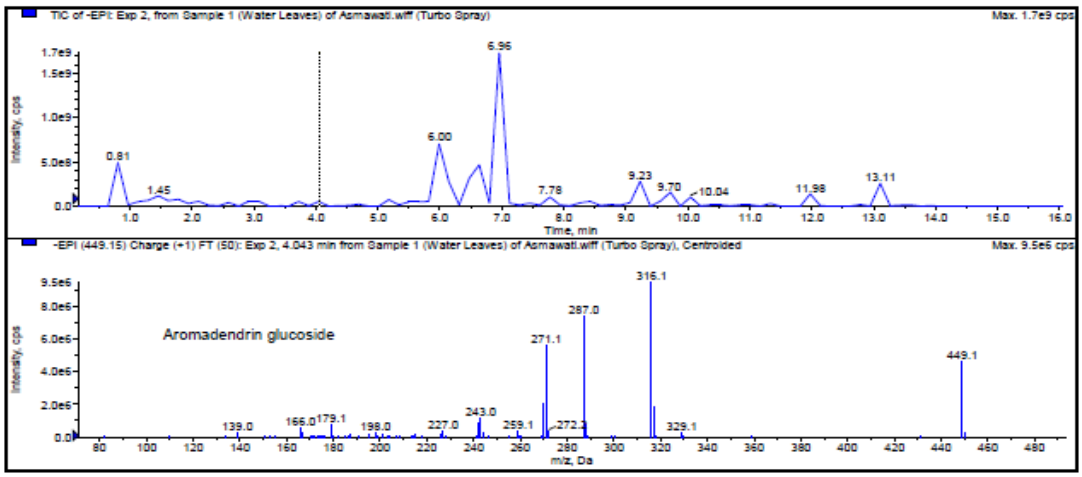

Figure 4.10 LCMS/MS chromatograms of aromadendrin glucoside from leaves water crude extract of *L. flavescens*

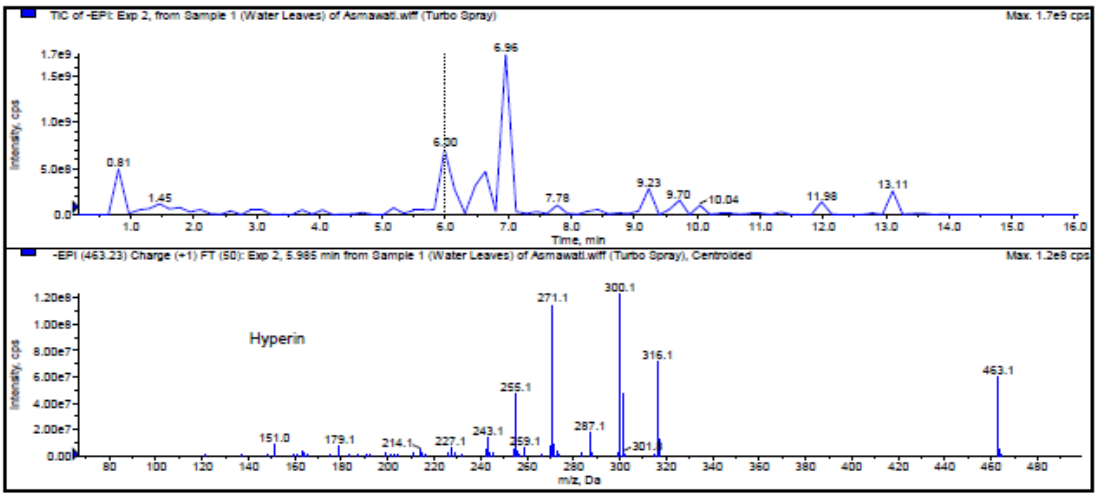

Figure 4.11 LCMS/MS chromatograms of hyperin from leaves water crude extract of *L. flavescens*

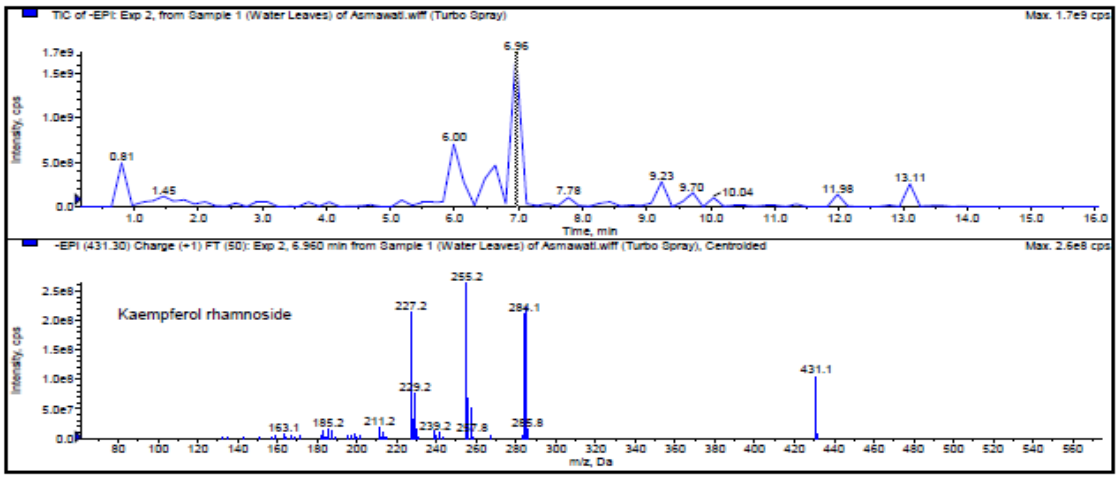

Figure 4.12 LCMS/MS chromatograms of kaempferol rhamnoside from leaves water crude extract of *L. flavescens*

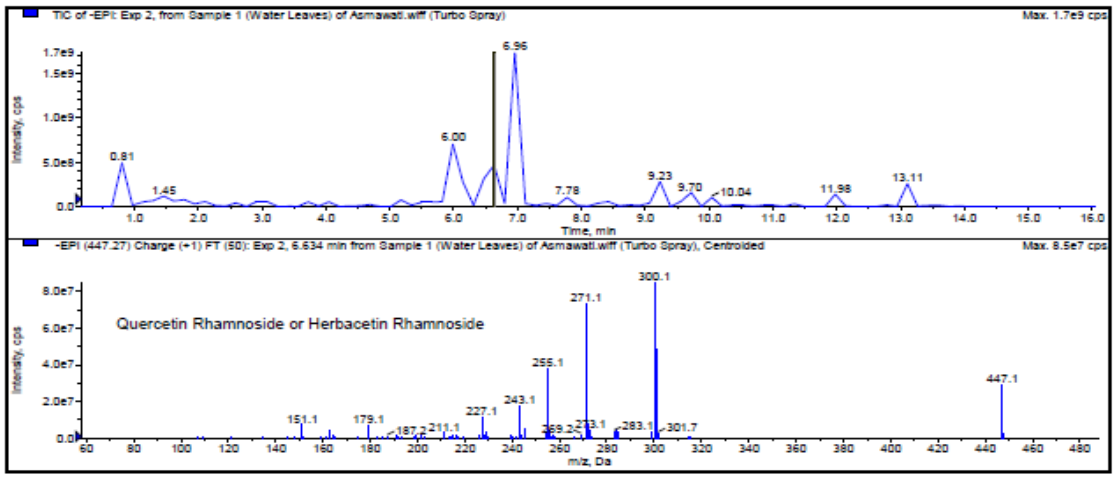

Figure 4.13 LCMS/MS chromatograms of quercetin or herbacetin rhamnoside from leaves water crude extract of *L. flavescens*

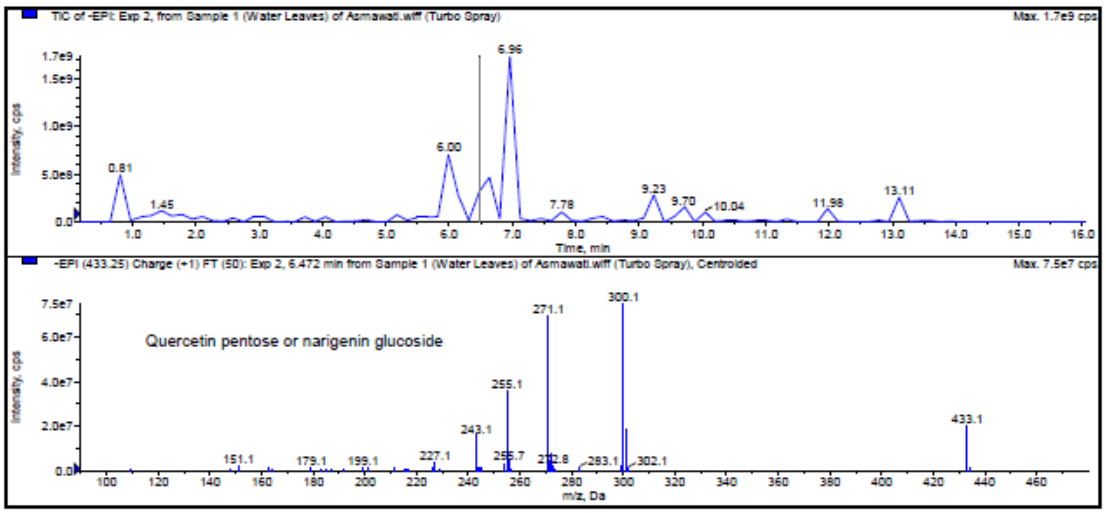

Figure 4.14 LCMS/MS chromatograms of narigenin from leaves water crude extract of *L. flavescens*

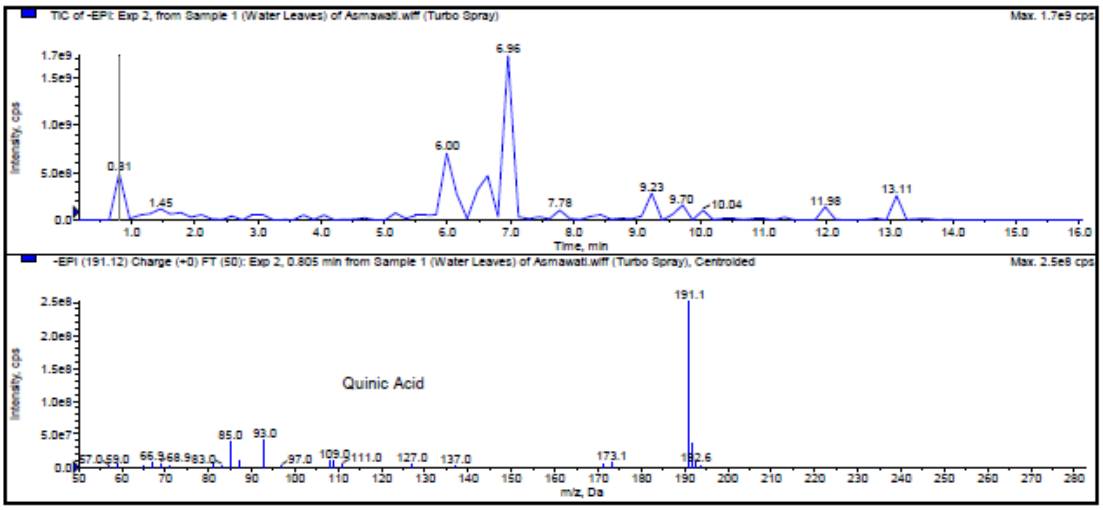

Figure 4.15 LCMS/MS chromatograms of quinic acid from leaves water crude extract of *L. flavescens*

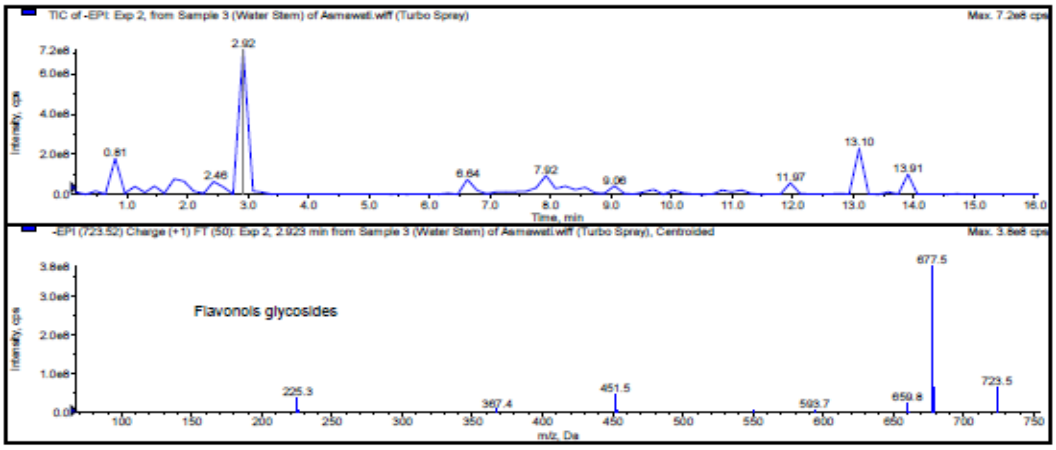

Figure 4.16 LCMS/MS chromatograms of flavonol glycosides from stems water crude extract of *L. flavescens*

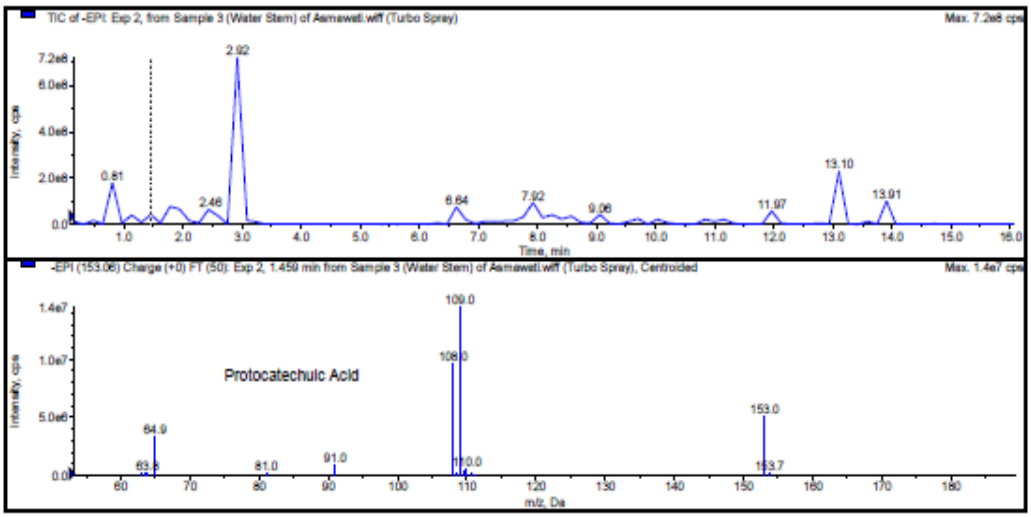

Figure 4.17 LCMS/MS chromatograms of protocatechuic acid from stems water crude extract of *L. flavescens*

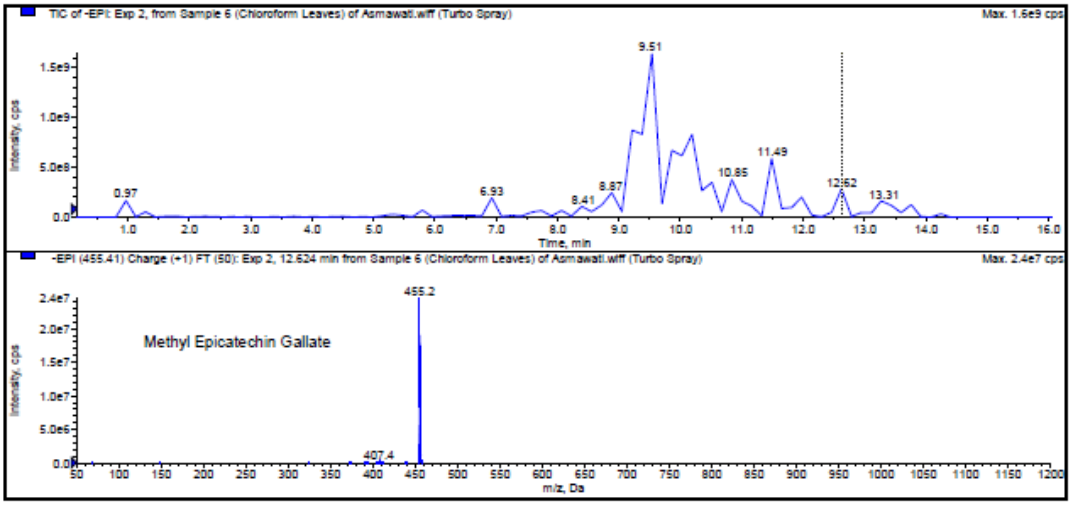

Figure 4.18 LCMS/MS chromatograms of methyl epicatechin gallate from leaves chloroform crude extract of *L. flavescens*

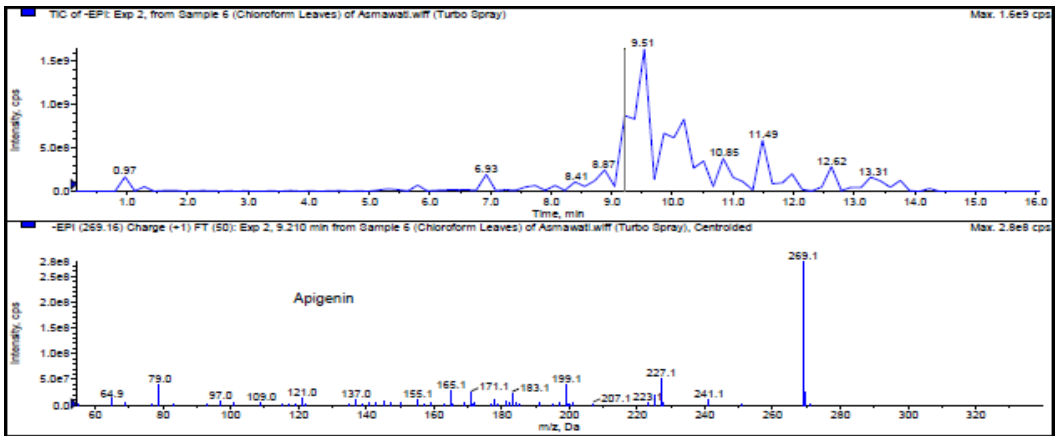

Figure 4.19 LCMS/MS chromatograms of apigenin from leaves chloroform crude extract of *L. flavescens*

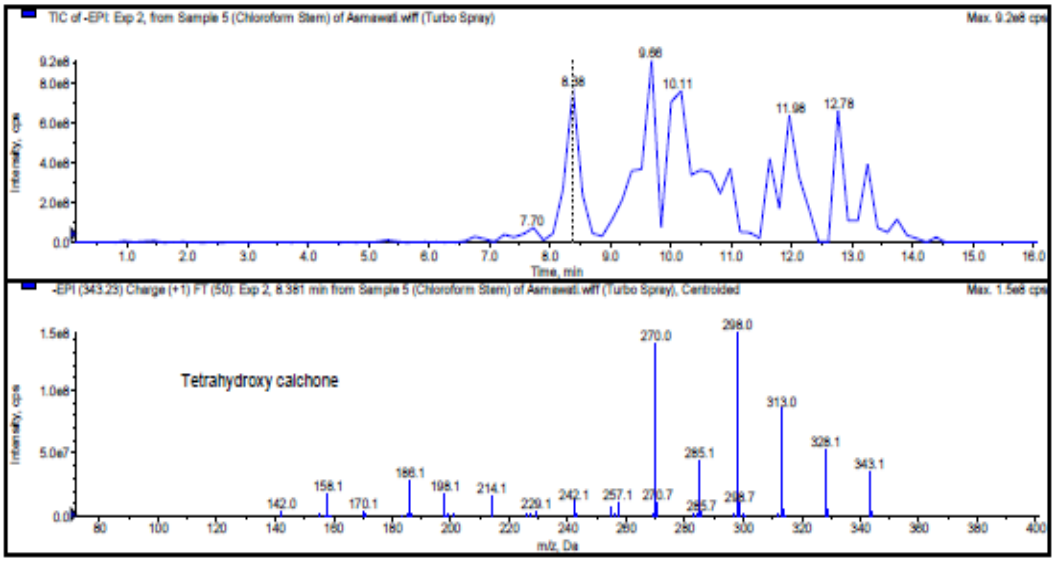

Figure 4.20 LCMS/MS chromatograms of tetrahydroxy chalcone from stems chloroform crude extract of *L. flavescens*

4.3 DETERMINATION OF TOTAL PHENOL CONTENT

Standard curve of gallic acid was developed to determine the total phenolic contents of hexane, chloroform and water extract from the leaves and stems of *L. flavescens* (Table 4.14). Figure 4.21 showed the standard curve of gallic acid. The phenol content of each extract was measured by using equation from the standard curve. Table 4.15 showed the total phenolic contents of crude hexane, chloroform and water extract from the leaves and stems of *L. flavescens*.

i) Standard phenol (gallic acid)

Table 4.14 Standard curve of gallic acid

Concentration gallic acid (mg/ml)	Absorbance at 765 nm		Mean ± S.D
	Replicate 1	Replicate 2	
2.5	1.981	1.955	1.968 ± 0.018
2.0	1.682	1.627	1.655 ± 0.039
1.5	1.239	1.105	1.172 ± 0.095
1.0	0.301	1.265	0.783 ± 0.682
0.5	0.491	0.499	0.495 ± 0.006

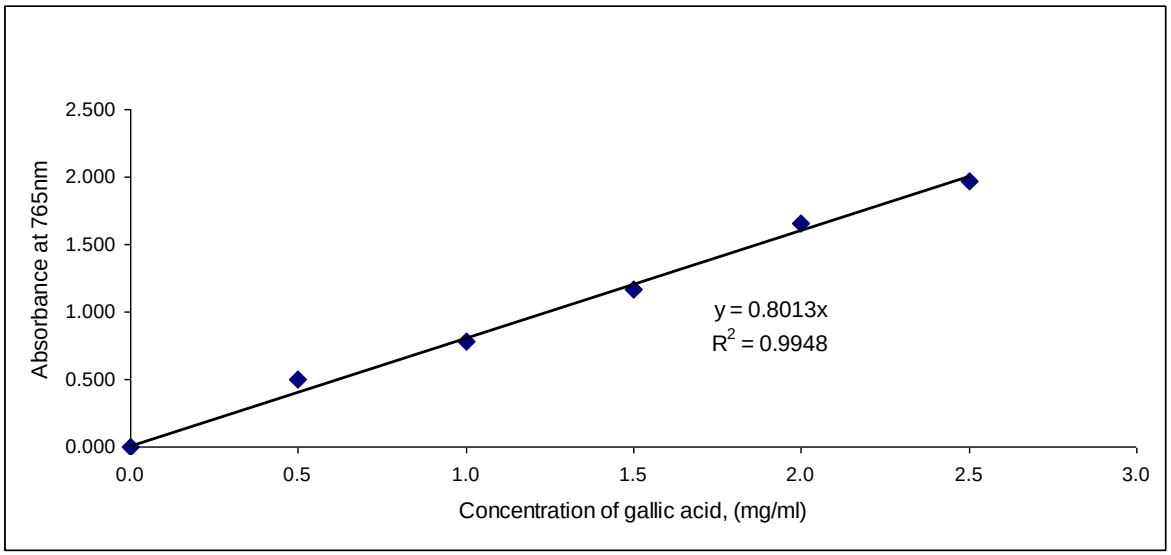

Figure 4.21 Standard curve of gallic acid

ii) Total phenolic of leaves and stems crude extracts of *L. flavescens*

Table 4.15 Total phenolic of crude extract from *L. flavescens*

Crude extract	Absorbance at 765 nm			Total Phenolic Content, (GAE mg/g dry mass)
	Replicate 1	Replicate 2	Mean ± S.D	
Leaves Hexane	0.246	0.261	0.254 ± 0.011	31.70
Stems Hexane	0.135	0.15	0.143 ± 0.011	17.85
Leaves Chloroform	0.344	0.343	0.344 ± 0.001	42.93
Stems Chloroform	0.154	0.162	0.158 ± 0.006	19.71
Leaves Water	1.362	1.259	1.311 ± 0.073	163.61
Stems Water	1.106	1.066	1.086 ± 0.028	135.53

4.4 DETERMINATION OF TOTAL FLAVONOID CONTENT

Standard curve of quarcetin was developed to determine the total flavonoid contents of hexane, chloroform and water extract from the leaves and stems of *L. flavescens* (Table 4.16). Figure 4.22 showed the standard curve of quarcetin. The flavonoid content of each extract was measured by using equation from the standard curve. Table 4.17 showed the total flavonoid contents of crude hexane, chloroform and water extract from the leaves and stems of *L. flavescens*.

i) Standard quarcetin

Table 4.16 Standard curve of quarcetin

Concentration quarcetin (mg/ml)	Absorbance at 510 nm		Mean ± S.D
	Replicate 1	Replicate 2	
2.5	0.198	0.199	0.199 ± 0.001
2.0	0.130	0.192	0.161 ± 0.044
1.5	0.115	0.124	0.119 ± 0.006
1.0	0.028	0.122	0.075 ± 0.066
0.5	0.058	0.038	0.048 ± 0.014

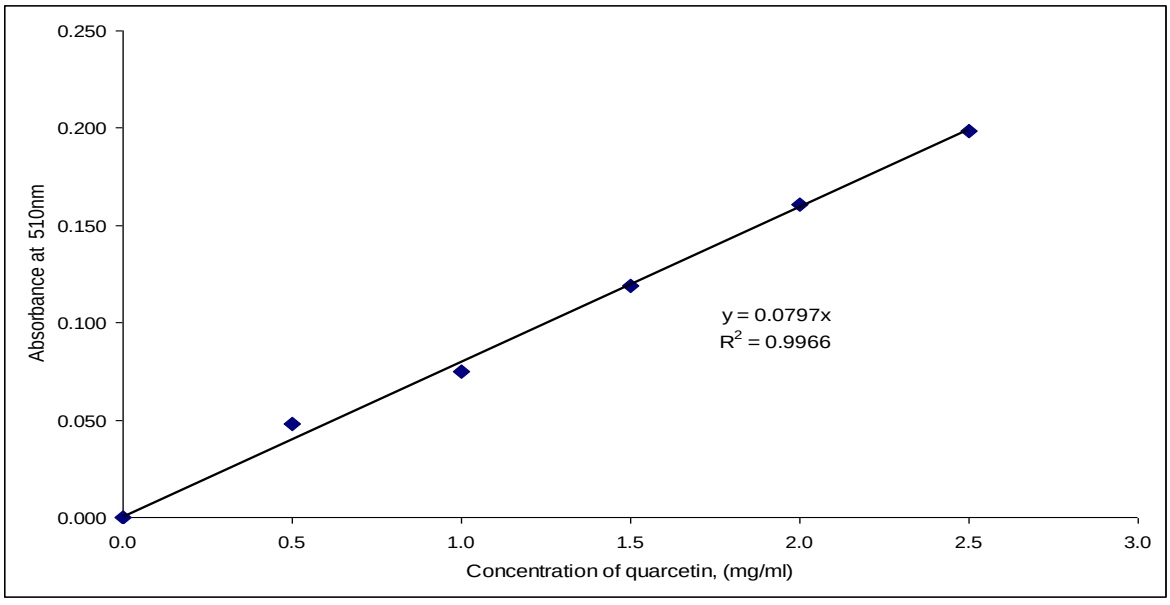

Figure 4.22 Standard curve of quarcetin

ii) Total flavonoid of leaves and stems crude extracts of *L. flavescens*

Table 4.17 Total flavonoid of crude extract from *L. flavescens*

Crude extract	Absorbance at 510nm			Total flavonoid Content (QE mg/g dry mass)
	Replicate 1	Replicate 2	Mean ± S.D	
Leaves Hexane	0.114	0.117	0.116 ± 0.002	145.55
Stems Hexane	0.094	0.09	0.092 ± 0.003	115.43
Leaves Chloroform	0.091	0.09	0.091 ± 0.001	114.18
Stems Chloroform	0.078	0.077	0.078 ± 0.001	97.87
Leaves Water	0.085	0.082	0.084 ± 0.002	105.39
Stems Water	0.096	0.094	0.095 ± 0.001	119.20

4.5 BRINE SHRIMP LETHALITY ASSAY (BSLA)

Table 4.18 showed the LC_{50} value of different crude extracts from *L. flavescens*. When the LC_{50} value was higher it meant that the toxicity of the crude extract was lower. The highest LC_{50} value was the chloroform extract from stems of *L. flavescens*, which was 609.14 μg/ml, while the lowest LC_{50} value was the chloroform extract from leaves with 21.54 μg/ml. This meant that 21.54 μg/ml was needed to inhibit the 50 % population of the brine shrimp. The probit analysis table of crude extract from leaves and stems of *L. flavescens* showed at Table 8.1 – Table 8.6.

Table 4.18 Number of dead shrimp in leaves and stems crude extract of *L. flavescens*

Concentration sample (μg / ml)	Total Number of Shrimp						Number of dead					
	Hexane		Chloroform		Water		Hexane		Chloroform		Water	
	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems
1000	10	10	10	10	10	10	9	9	6	7	9	9
100	10	10	10	10	10	10	4	1	5	0	2	0
10	10	10	10	10	10	10	1	0	5	1	3	1

Table 4.19 showed the total phenolic contents, total flavonoid contents and LC_{50} value of crude hexane, chloroform and water from the leaves and stems of *L. flavescens*.

Table 4.19 Total phenolic content, total flavonoid content and LC_{50} of leaves crude extract of *L. flavescens*

Sample	Total Phenol Content (GAE mg/g Dry mass)	Total Flavonoid Content (QE mg/g Dry mass)	LC_{50} (µg/ml)
Leaves Hexane	31.70	145.55	124.23
Stems Hexane	17.85	115.43	316.33
Leaves Chloroform	42.93	114.18	21.54
Stems Chloroform	19.71	97.87	609.14
Leaves Water	163.61	105.39	124.57
Stems Water	135.53	119.20	295.65

4.6 ANTIDIABETIC ACTIVITY BIOASSAY – HYPERGLYCEMIA ASSAY

Glycogen phosphorylase (GP) an activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate. And then the phosphate absorbance was measured at 655 nm.

i) Determination of GP inhibition using Caffeine as standard

Caffeine was used as the standard in the Glycogen phosphorylase (GP) inhibitory assay. The ability of the Caffeine acted as GP inhibitor to inhibit the process of glycogen degradation and reduce hepatic glucose production (HGP). Various concentration of Caffeine were tested; 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml (Table 4.20). At the concentration of 0.6 mg/ml, the percentage of GP inhibitor was 97 %.

ii) Determination of GP inhibition of crude extracts of *L. flavescen*s

The GP inhibitory activities of hexane, chloroform and water extracts from the leaves and stems of *L. flavescens* were determined (Table 4.20). All the crude extract with 5 different concentrations was also tested by using GP inhibitory activity to measure the percentage inhibition of GP activity. The percentage of GP inhibition increased when the concentration of test samples increased. Figure 4.23 showed the GP inhibition of leaves and stems extract of *L. flavescens* and Caffeine as standard.

Table 4.20 Percentage of glycogen phosphorylase inhibition in leaves and stems extract of *L. flavescens* and Caffeine as standard.

Samples	Concentration (mg/ml)	% GP inhibition
Standard (Caffeine)	0.2	87.56
	0.4	95.52
	0.6	96.51
	0.8	95.52
	1.0	95.52
Leaves Hexane (LH)	0.2	74.62
	0.4	82.58
	0.6	83.58
	0.8	82.58
	1.0	81.59
Leaves Chloroform (LC)	0.2	69.65
	0.4	80.59
	0.6	81.59
	0.8	80.59
	1.0	79.60
Leaves Water (LD)	0.2	79.60
	0.4	86.56
	0.6	86.56
	0.8	86.56
	1.0	85.57
Stems Hexane (SH)	0.2	59.70
	0.4	64.67
	0.6	64.67
	0.8	64.67
	1.0	64.67
Stems Chloroform (SC)	0.2	49.75
	0.4	54.72
	0.6	55.72
	0.8	56.71
	1.0	56.71
Stems Water (SD)	0.2	39.80
	0.4	44.77
	0.6	44.77
	0.8	44.77
	1.0	44.77

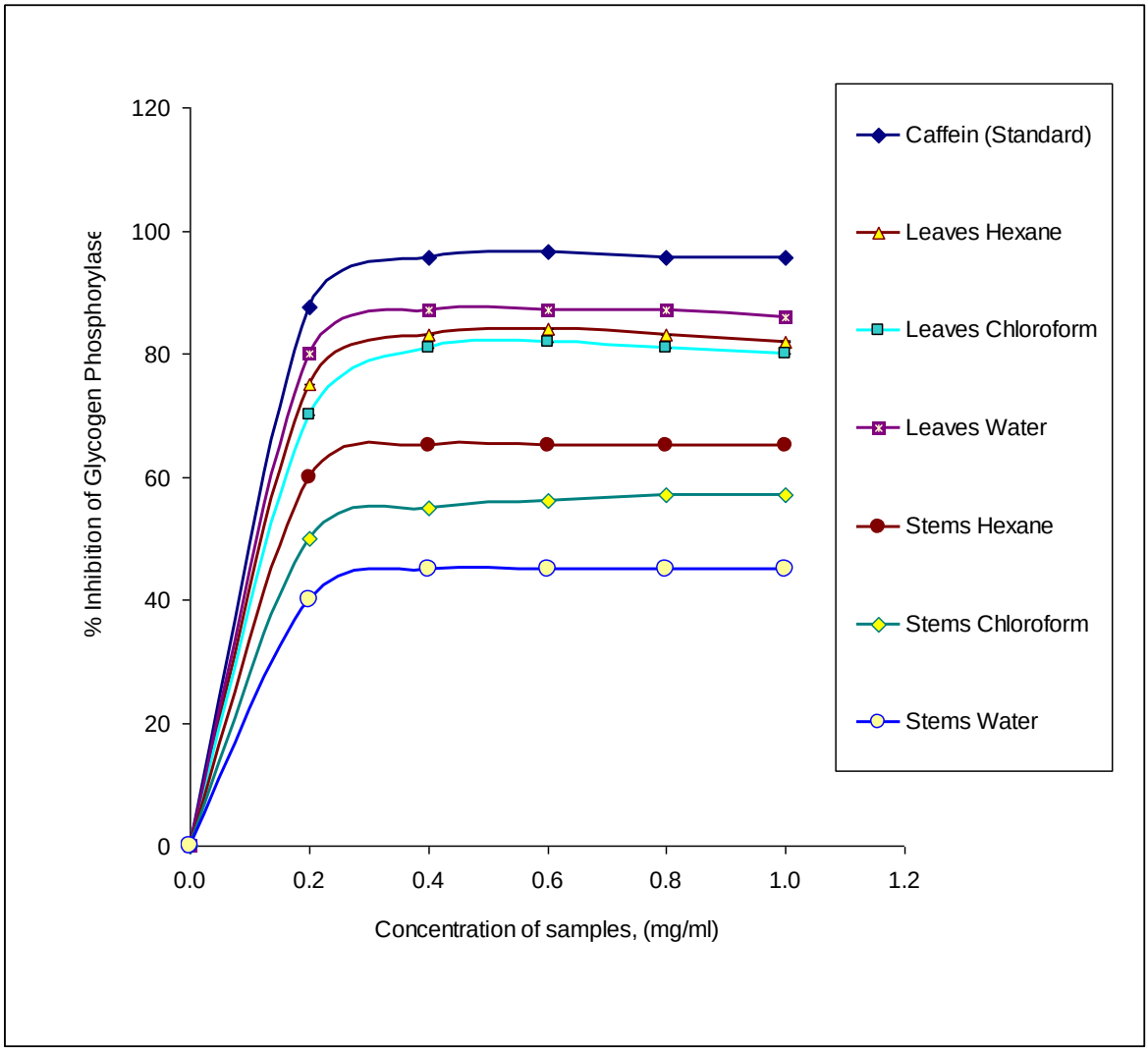

Figure 4.23 Inhibition of glycogen phosphorylase of crude extract by *L. flavescens*

4.7 ANIMAL STUDY

4.7.1 Determination of oral glucose tolerance test (OGTT)

Figure 4.24 depicts the hypoglycemic effects of single oral administration of the extracts dose at 0.2 g/kg and 0.5 g/kg on OGTT of normal rats. The leaves and stems water extract dose at 0.2 g/kg and 0.5 g/kg produced a maximum fall at 60 minutes after glucose administration (Table 8.9-Table 8.14).

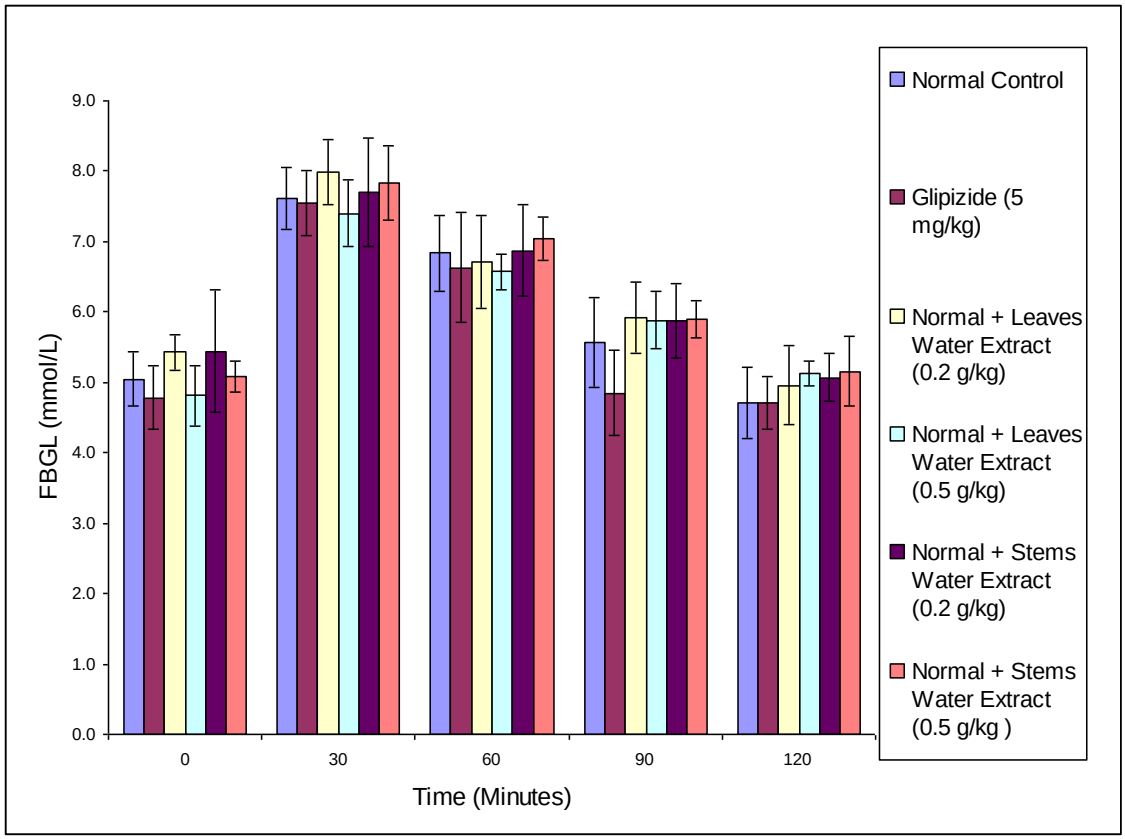

Figure 4.24 Hypoglycemic effect of water extract of *Leptospermum flavescens* on fasting blood glucose level (FBGL) of normal rats during OGTT

* Each value shown in mean ± S.E. n (number of animals in each group) = 6.

4.7.2 Determination of acute toxicity of *Leptospermum flavescens*

Acute toxicity study revealed the non-toxic nature of the extracts. There was no lethality or any toxic reactions found at any of the doses selected until the end of the study period. The water extract of *Leptospermum flavescens* did not show any mortality and none of the treated rats showed any visible symptoms of toxicity up to a dose of 0.5 g/kg body weight. Even at this high dose these was no gross behavioral changes indicating high margins of safety (Table 8.15- Table 8.20).

4.7.3 Experimental induction of non-insulin dependent diabetes mellitus (NIDDM)

After 72 hours NIDDM induction, overnight fasted adult male Sprague–Dawley (SD) rats weighing (150 g –200 g) become hyperglycaemic which is confirmed by the elevated glucose levels determined. Animals with blood glucose level more than 150 mg/dl or 8.3 mmol/L were considered as diabetic. Rats found with permanent NIDDM were used for the antidiabetic study. Plasma level of sugar (glucose) is the main criterion in diagnostics and follows up of diabetes mellitus. In this study, units of mmol/L (milimol per litre) are used.

4.7.4 Measurement of fasting blood glucose level (FBGL)

After 20 days of treatment with *L. flavescens* water extract, maximum reduction in plasma blood glucose level was observed in diabetic rats treated with leaves and stems of water extract from *L. flavescens* dose at 0.2 g/kg and 0.5 g/kg body weight (Table 4.21). Administration of the *L. flavescens* water extract for a long duration led to a significant diminution of fasting blood glucose (FBG) in the diabetic rats, while there was no significant alteration in the FBG of the control animals. Leaves of water extract from *L. flavescens* dose at 0.2 g/kg body weight significantly decreased the FBG of the

diabetic rats from an initial level of 14.45 ± 0.11, 13.29 ± 0.25, 11.57 ± 0.16, 10.82 ± 0.20 and 8.53 ± 0.19 mmol/L on 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day, respectively. Leaves of water extract from *L. flavescens* dose at 0.5 g/kg body weight significantly decreased the FBG of the diabetic rats from an initial level of 14.28 ± 0.10, 12.22 ± 0.09, 8.28 ± 0.12, 6.85 ± 0.10 and 5.12 ± 0.14 mmol/L on 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day, respectively. Stems of water extract from *L. flavescens* dose at 0.2 g/kg body weight significantly decreased the FBG of the diabetic rats from an initial level of 14.13 ± 0.21, 13.73 ± 0.18, 13.52 ± 0.19, 10.90 ± 0.39 and 8.69 ± 0.21 mmol/L on 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day, respectively. Stems of water extract from *L. flavescens* dose at 0.5 g/kg body weight significantly decreased the FBG of the diabetic rats from an initial level of 14.65 ± 0.10, 13.85 ± 0.26, 9.60 ± 0.13, 7.03 ± 0.15 and 5.18 ± 0.30 mmol/L on 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} day, respectively. The FBG became normal by day 20^{th} (Table 4.21).

Table 4.21 Effect of *L. flavescens* extracts on fasting blood glucose level (FBGL) in diabetic rats.

Group, n = 6	Treatment	Fasting plasma glucose concentration, (mmol/L)				
		0th day	5th day	10th day	15th day	20th day
I	Normal control	6.05 ± 0.21	5.9 ± 0.06	5.55 ± 0.18	5.15 ± 0.11	5.07 ± 0.26
II	Diabetic control	14.88 ± 0.05	14.65 ± 0.12	14.32 ± 0.03	14.07 ± 0.24	13.72 ± 0.24
III	Glipizide (5 mg/kg)	14.72 ± 0.11	13.22 ± 0.08	10.52 ± 0.06	8.72 ± 0.3	7.78 ± 0.18
IV	Diabetic + Leaves Water Extract (0.2 g/kg)	14.45 ± 0.11	13.29 ± 0.25	11.57 ± 0.16	10.82 ± 0.20	8.53 ± 0.19
V	Diabetic + Leaves Water Extract (0.5 g/kg)	14.28 ± 0.10	12.22 ± 0.09	8.28 ± 0.12	6.85 ± 0.10	5.12 ± 0.14
VI	Diabetic + Stems Water Extract (0.2 g/kg)	14.13 ± 0.21	13.73 ± 0.18	13.52 ± 0.19	10.90 ± 0.39	8.69 ± 0.21
VII	Diabetic + Stems Water Extract (0.5 g/kg)	14.65 ± 0.10	13.85 ± 0.26	9.60 ± 0.13	7.03 ± 0.15	5.18 ± 0.30

The data of FBGL measurement were statistically evaluated using two-way ANOVA, followed by Turkey and Games-Howell test using SPSS 14.0 software. The values were considered statistically significant when $p<0.05$ level. All the results were expressed as mean ± S.E.

4.7.5 Biochemical analysis : estimation of lipid profile and liver function

The effect of the administration of leaves and stems from *L. flavescens* water extract for 20 days on total lipid profile and liver function is shown in Table 4.22 & Table 4.23 respectively. A decrease in the serum triglycerides, total cholesterol, LDL (low density lipids) and increase in the HDL (high density lipids) cholesterol levels were observed. The activities of ALT, AST, ALP and total protein are content in liver of normal and diabetic rats also were observed.

A significant increase ($p<0.05$) in the activities of ALT, AST, ALP and total protein content were observed in diabetic rats. Oral administration of *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and Glipizide dose at 5 mg/kg body weight reversed these activities to near normal. There was a significant elevation in triglycerides, total cholesterol, LDL cholesterol, ALT, AST, ALP and total protein in the diabetic rats.

The administration of the leaves *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight administration of the stems *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and Glipizide dose at 5 mg/kg body weight tended to bring triglycerides ($p<0.05$), total cholesterol ($p<0.05$), HDL cholesterol ($p<0.05$), LDL cholesterol ($p<0.05$), ALT ($p<0.05$), AST ($p<0.05$), ALP ($p<0.05$) and total protein ($p<0.05$) significantly toward normal values, while normal rats did not exhibit any significant alterations in these parameters during the experiment. The leaves and stems *L. flavescens* water extract treatment groups were found to have similar effects compared to Glipizide.

Total cholesterol in the diabetic control group was significantly higher (1.85 ± 0.09 mmol/L) compared to (1.60 ± 0.07 mmol/L) in the healthy control, which remained high at the end of the experiment on 20th day. However, total cholesterol significantly decreased to 1.55 ± 0.06 mmol/L, 1.40 ± 0.09 mmol/L, 1.37 ± 0.11 mmol/L, 1.43 ± 0.11 mmol/L and 1.40 ± 0.11 mmol/L on 20^{th} day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves of *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and the stems of *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg, body weight respectively.

Similarly LDL cholesterol values in the diabetic control group showed increase on 20 days. LDL increased to 0.88 ± 0.08 mmol/L compared to (0.63 ± 0.16) normal control on 20th day. In the treated group, LDL level was significantly decreased to 0.80 ± 0.15 mmol/L, 0.25 ± 0.11 mmol/L, 0.21 ± 0.09 mmol/L, 0.48 ± 0.12 mmol/L and 0.39 ± 0.14 mmol/L on 20th day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves of *L. flavescens* water extract dose at 0.2 and 0.5 g/kg body weight and the stems of *L. flavescens* water extract dose at 0.2 and 0.5 g/kg body weght respectively on 20th day. Likewise, the increased triglyceride levels were also brought down close to the normal values by administration of the extract for 20 days. The value decreased to 0.76 ± 0.18 mmol/L, 0.52 ± 0.10 mmol/L, 0.53 ± 0.08 mmol/L, 0.45 ± 0.04 mmol/L and 0.47 ± 0.18 mmol/L on 20th day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves of *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and the stems of *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight respectively on 20th day of the experiment.

In contrast, HDL cholesterol was significantly decreased in the diabetic control group as compared to the normal control, and was further decreased to 0.52 ± 0.05 mmol/L on

20th day. But, treatment with the extract caused significant improvement and increased the level to 1.05 ± 0.13 mmol/L, 1.15 ± 0.10 mmol/L, 1.21 ± 0.19 mmol/L, 0.66 ± 0.02 mmol/L and 1.07 ± 0.19 mmol/L on 20th day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves of *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and the stems of *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight respectively on 20th day of the experiment. No significant change occurred in the lipid profile of the animals in the normal control group during the experimental period.

The activities of ALT, AST, ALP and total protein content were observed. ALT in the diabetic control group was significantly higher (199.17 ± 58.64 IU/L) compared to (156.00 ± 14.18 IU/L) in the healthy control, which remained high at the end of the experiment on 20th day. However, ALT significantly decreased to 109.33 ± 14.41 IU/L, 83.50 ± 6.61 IU/L, 80.00 ± 12.77 IU/L, 103.00 ± 14.19 IU/L and 85.83 ± 9.82 IU/L on 20th day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and the stems *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight respectively.

AST in the diabetic control group was significantly higher (689.50 ± 263.13 IU/L) compared to (353.83 ± 74.59 IU/L) in the healthy control, which remained high at the end of the experiment on 20th day. However, AST significantly decreased to 257.50 ± 45.74 IU/L, 223.33 ± 55.83 IU/L, 154.67 ± 10.06 IU/L, 245.67 ± 47.18 IU/L and 243.33 ± 43.23 IU/L on 20th day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and of the stems *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight respectively.

ALP in the diabetic control group was significantly higher (868.00 ± 142.92 IU/L) compared to (378.00 ± 60.26 IU/L) in the normal control, which remained high at the end of the experiment on 20th day. However, ALT significantly decreased to 366.00 ± 71.71 IU/L, 210.83 ± 22.68 IU/L, 147.00 ± 27.14 IU/L, 345.50 ± 74.04 IU/L and 286.33 ± 63.20 IU/L on 20th day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and of the stems *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight respectively.

Total protein in the diabetic control group was significantly higher (70.17 ± 1.25 g/L) compared to (70.17 ± 1.25 g/L) in the healthy control, which remained high at the end of the experiment on 20th day. However, total protein significantly decreased to 67.67 ± 2.35 g/L, 62.33 ± 5.85 g/L, 61.67 ± 4.42 g/L, 66.33 ± 0.76 g/L and 64.50 ± 3.94 g/L on 20th day of treatment with the Glipizide dose at 5 mg/kg body weight, leaves *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and of the stems *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight respectively.

Table 4.22 The effect of extract of *L. flavescens* on serum lipid profile in diabetic rats.

Group n = 6	Treatment	Serum Lipid Profile Test (mmol/L)			
		Triglyceride (mmol/L)	Total Cholesterol (mmol/L)	HDL Cholesterol (mmol/L)	LDL Cholesterol (mmol/L)
I	Normal control	0.90 ± 0.21	1.60 ± 0.07	1.10 ± 0.17	0.63 ± 0.16
II	Diabetic control	1.09 ± 0.02	1.85 ± 0.09	0.52 ± 0.05	0.88 ± 0.08
III	Glipizide (5 mg/kg)	0.76 ± 0.18	1.55 ± 0.06	1.05 ± 0.13	0.80 ± 0.15
IV	Diabetic + Leaves Water Extract (0.2 g/kg)	0.52 ± 0.10	1.40 ± 0.09	1.15 ± 0.10	0.25 ± 0.11
V	Diabetic + Leaves Water Extract (0.5 g/kg)	0.53 ± 0.08	1.37 ± 0.11	1.21 ± 0.19	0.21 ± 0.09
VI	Diabetic + Stems Water Extract (0.2 g/kg)	0.45 ± 0.04	1.43 ± 0.11	0.66 ± 0.02	0.48 ± 0.12
VII	Diabetic + Stems Water Extract (0.5 g/kg)	0.47 ± 0.18	1.40 ± 0.11	1.07 ± 0.19	0.39 ± 0.14

Table 4.23 The effect of extract of *L. flavescens* on liver function in diabetic rats

Group n = 6	Treatment	Liver Function Test (mmol/L)			
		ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total Protein (g/L)
I	Normal control	156.00 ± 14.18	353.83 ± 74.59	378.00 ± 60.26	68.83 ± 1.05
II	Diabetic control	199.17 ± 58.64	689.50 ± 263.13	868.00 ± 142.92	70.17 ± 1.25
III	Glipizide (5 mg/kg)	109.33 ± 14.41	257.50 ± 45.74	366.00 ± 71.71	67.67 ± 2.35
IV	Diabetic + Leaves Water Extract (0.2 g/kg)	83.50 ± 6.61	223.33 ± 55.83	210.83 ± 22.68	62.33 ± 5.85
V	Diabetic + Leaves Water Extract (0.5 g/kg)	80.00 ± 12.77	154.67 ± 10.06	147.00 ± 27.14	61.67 ± 4.42
VI	Diabetic + Stems Water Extract (0.2 g/kg)	103.00 ± 14.19	245.67 ± 47.18	345.50 ± 74.04	66.33 ± 0.76
VII	Diabetic + Stems Water Extract (0.5 g/kg)	85.83 ± 9.82	243.33 ± 43.23	286.33 ± 63.20	64.50 ± 3.94

Note:

BW =Body weight, HDL= High-density lipoprotein, LDL = Low-density lipoprotein, ALT = alanine aminotransferase, AST = aspartate aminotransferase, ALP = alkaline phosphatase

Data were statistically evaluated using one-way ANOVA for biochemical analysis to estimation of lipid profile and liver function, followed by Turkey and Games-Howell test using SPSS 14.0 software. The values were considered statistically significant when $p<0.05$ level. All the results were expressed as mean ± S.E.

4.7.6 Measurement of changes in body weight.

After 20 days of treatment with extract, a gain in body weight was observed in treated rats as compared to diabetic control rats and normal control rats in Figure 4.25. Loss of body weight is a characteristic condition in diabetes, owing to defect in glucose metabolism and excessive breakdown of tissue protein. After 20 days of treatment, normal control group, *L. flavescens* water extract treated groups and Glipizide treated groups increased significantly ($p<0.05$) in body weight, when compared to day 0. The Alloxan induced diabetic rats had significantly lost body weight, compared to the normal control (Figure 4.25).

The effect of *L. flavescens* water extract on body weight, physiological parameters such as body weight of rats in the control and treatment group are summarized in Table 8.35 - Table 8.41 While the body weight of the rats in the normal control group increased significantly from 0.155 ± 0.0001 kg on 0 day to 0.211 ± 0.003 kg on 20th day, there was no appreciable increase in the diabetic controls. On the other hand, *L. flavescens* water extract treated rats gained significant body weight during the treatment period. Their body weight increased to 0.189 ± 0.002 kg on 20th day from 0.159 ± 0.001 kg an initial weight of at the beginning of treatment with the Glipizide dose at 5 mg/kg body weight, increased to 0.194 ± 0.001 kg on 20th day from 0.157 ± 0.001 kg an initial weight of at the beginning of treatment with the leaves *L. flavescens* water extract dose at 0.2 g/kg body weight, increased to 0.197 ± 0.001 kg on 20th day from 0.159 ± 0.001 kg an initial weight of at the beginning of treatment with the leaves *L. flavescens* water extract dose at 0.5 g/kg body weight, increased to 0.195 ± 0.001 kg on 20th day from 0.156 ± 0.001 kg an initial weight of at the beginning of treatment with the stems *L. flavescens* water extract dose at 0.2 g/kg body weight, and also increased to 0.194 ±

0.001 kg on 20th day from 0.154 ± 0.001 kg an initial weight of at the beginning of treatment with the stems *L. flavescens* water extract dose at 0.5 g/kg body weight. Figure 4.25 represents the changes in body weight in normal and experimental diabetic rats. Alloxan produced significant loss in body weight as compared to normal rats during the study. Diabetic control continued to lose weight till the end of the study while the leaves *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight and the stems *L. flavescens* water extract dose at 0.2 g/kg and 0.5 g/kg body weight respectively showed significant difference in body weight compared to diabetic control group. There was no significant difference between the *L. flavescens* water extract and Glipizide treated groups the diabetic rats treated with the extracts showed can control in the loss of body weight as compared to diabetic control group. While the *L. flavescens* water extract treated groups the diabetic rats treated showed a better control in the loss of body weight.

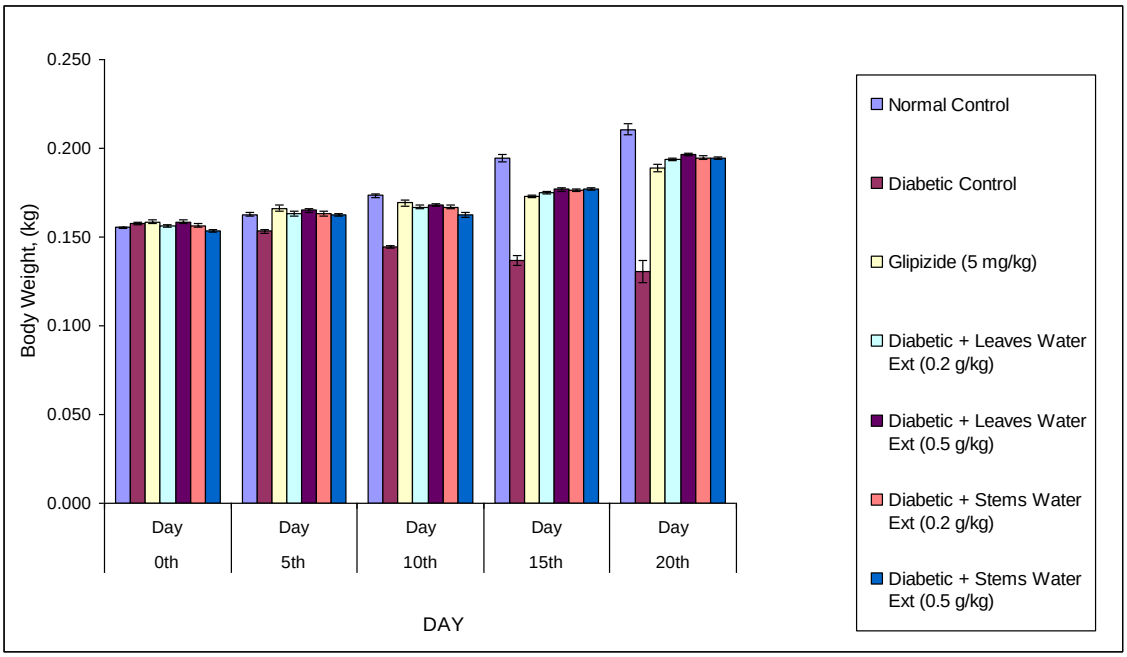

Figure 4.25 Effect of water extract from *L. flavescens* on body weight of diabetic rats

The data of changes in body weight were statistically evaluated using two-way ANOVA, followed by Turkey and Games-Howell test using SPSS 14.0 software. The values were considered statistically significant when $p<0.05$ level. All the results were expressed as mean ± S.E.

CHAPTER 5

5.0 DISCUSSIONS

The dried-powdered leaves and stems of *Leptospermum flavescens* were extracted by using hexane, chloroform and water. The polar solvent will extracted out the polar compound and the non-polar compound will be extracted by the non-polar solvent. Therefore, the non-polar solvent was used and the only force presented between molecules was dispersion, because of the production of transient charges induced in the individual molecules. Non polar solvents consisting of disordered molecules allowed the introduction of other non polar molecules easily. In this experiment, the hexane dissolved the non polar compounds such as fats and waxes, while polar solvents such as water dissolved the polar compounds such as alkaloid (Ross & Brain, 1977).

After extraction, separation of chemical compound from the leaves and stems crude extract of *L. flavescens* were done by using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry combined with mass spectrometry (LCMS/MS). TLC was one separation technique that was inexpensive and quick technique. The advantages of this technique are short separation time amenability to detection reagents and the possibility of running several samples at the same time. TLC also ideally suited for the preliminary screening of plant extracts before HPLC analysis.

A large number of solvent systems were tried to achieve a good resolution. Finally the solvent system methanol: chloroform (10:90, v/v) and toluene: ethyl acetate (93:7, v/v) was selected for this crude extract of *L. flavescens*. This mixture is strong enough to move the bottom sample significantly from the sample line. Major spots in this TLC

analysis showed flavonoid compounds found in crude extract of *L. flavescens*. Other compounds were found such alkaloid, saponin, terpenoid, and essential oil. As with selectivity each solvent has its own polarity and each solvent mixture or mobile phase then has its own unique solvent strength. The dilution of a solvent mixture with a less polar solvent from 60 % to 99 % can reduce solvent strength, increasing compound retention and resolution (Ali, *et al.*, 2011).

In this study, dilution of methanol polar solvent with 90 % chloroform as a less polar solvent and dilution of ethyl acetate polar solvent with 93 % toluene as a less polar solvent have been selected as solvent system in this TLC analysis. Both solvent systems have different solvent strength of 0.46 and 0.68 respectively.

TLC plates were sprayed with Dragendorff reagent and orange compound appeared indicated the presence of alkaloids. LHA17 labeled compound from the leaves of hexane extract by using Methanol: Chloroform (10: 90, v/v) as a solvent system (Table 4.1), LHC22 labeled compound from the leaves of hexane extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.2), LCA20 labeled compound from the leaves of chloroform extract by using Methanol: Chloroform (10: 90, v/v) as a solvent system (Table 4.3), LCC1, LCC16 labeled compound from the leaves of chloroform extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.4), LDC14, labeled compound from the leaves of water extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.6), SHC19, labeled compound from the stems of hexane extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.8), SCC4, labeled compound from the stems of chloroform extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.10), SDA4, labeled compound from the stems of water extract by using Methanol:

Chloroform (10: 90, v/v) as a solvent system (Table 4.11), SDC12, labeled compound from the stems of water extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.12) were observed as alkaloid.

The TLC plates were sprayed with Vanillin reagent and heated, if purple band or spot appeared, it indicated the presence of terpenoid. The labeled compounds; LHC15, LHC18, LHC22 from the leaves of hexane extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.2), the labeled compounds; LCA10, LCA11 from the leaves of chloroform extract by using Methanol: Chloroform (10: 90, v/v) as a solvent system (Table 4.3), the labeled compounds; LCC15 from the leaves of chloroform extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.4), the labeled compounds; LDA12, LDA15 from the leaves of water extract by using Methanol: Chloroform (10: 90, v/v) as a solvent system (Table 4.5), the labeled compounds; SHA7, SHA9 from the stems of hexane extract by using Methanol: Chloroform (10: 90, v/v) as a solvent system (Table 4.7), the labeled compounds; SHC12, SHC13, SHC15, SHC16, SHC18 from the stems of hexane extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.8), the labeled compounds; SCC10 from the stems of chloroform extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.10), the labeled compounds; SDA4, SDA6, SDA8 from the stems of water extract by using Methanol: Chloroform (10: 90, v/v) as a solvent system (Table 4.11) and the labeled compounds; SDC5, SDC6, SDC8, SDC9, SDC11 from the stems of water extract by using Toluene: Ethyl acetate (93: 7, v/v) as a solvent system (Table 4.12) were observed as Terpenoid.

The TLC plates were sprayed with Anisaldehyde reagent and if blue band appeared indicated the presence of essential oil. If black band appeared, it indicated the presence

of saponin and if red or other band appeared indicated the presence of flavonoid. LHA5 labeled compound from leaves of hexane crude extract by using (10: 90, v/v) Methanol: Chloroform as a solvent system (Table 4.1), LHC8, LHC10, LHC13, LHC16, LHC17, LHC18 labeled compound from leaves of hexane crude extract by using (93: 7, v/v) Toluene: Ethyl acetate as a solvent system (Table 4.2), LCC6, LCC9 labeled compound from leaves of chloroform crude extract by using (93: 7, v/v) Toluene: Ethyl acetate as a solvent system (Table 4.4), LDA5, LDA7, LDA10, LDA13, LDA14, LDA15 labeled compound from leaves of water crude extract by using 10: 90, Methanol: Chloroform as a solvent system (Table 4.5), LDC2 labeled compound from leaves of water crude extract by using (93: 7, v/v) Toluene: Ethyl acetate as a solvent system (Table 4.6), SHA10 labeled compound from stems of hexane crude extract by using (10: 90, v/v) Methanol: Chloroform as a solvent system (Table 4.7), SHC7, SHC9 labeled compound from stems of hexane crude extract by using (93: 7, v/v) Toluene: Ethyl acetate as a solvent system (Table 4.8), SDA9 labeled compound from stems of water crude extract by using (10: 90, v/v) Methanol: Chloroform as a solvent system (Table 4.11), SDC2 labeled compound from stems of water crude extract by using (93: 7, v/v) Toluene: Ethyl acetate as a solvent system (Table 4.12) were observed as saponin.

Other compounds were appeared as flavonoid was separated as labeled compound (Table 4.1 – Table 4.12). Most of band or spot appeared were detected as flavonoid compounds. The presence of alkaloids, flavonoids, saponins, terpernoids, and essential oil in *L. flavescens* crude extract may be having shown antidiabetic potential. Hence further separation of chemical compounds analysis such high performance liquid chromatography (HPLC) need to confirm the presence of desired compounds including separation, identification, purification, and quantification of various undesired compounds.

High performance liquid chromatography (HPLC) was a highly improved form of column chromatography. It was performed under high pressure and the detection method in HPLC was highly automated and very sensitive. From the previous study, it was reported that flavesone, leptospermone and isoleptospermone as antimicrobial components were presented in this plant (Mustafa, *et al.*, 2003). The important biological activities of the volatile oils produced by *L. flavescens* species of the Myrtaceae (Cheng, *et al.*, 2009), presented as a major component the sesquiterpene nerolidol and the content of other components such as α-pinene, β-pinene, *γ*-terpinene, 1, 8-cineole and terpinen-4-ol. In this study, we used HPLC to separate the chemical compounds that present in the leaves and stems crude extract of *L. flavescens* such phenolic and flavonoid compounds.

From Figure 4.1, HPLC chromatograms were observed and 19 compounds were separated from leaves chloroform crude extract of *L. flavescens* within 60 minutes. Figure 4.2, Figure 4.3, Figure 4.4, showed HPLC detected 10 chemicals compounds from the leaves water crude extract, 17 compounds were separated from the stems chloroform crude extract and 3 compounds were detected from the stems water crude extract of *L. flavescens* respectively.

HPLC analysis showed less chemical compounds compared with thin layer chromatography analysis. The peaks of chemical compounds were confirmed by comparing of their retention time with standard. In this study quarcetin, gallic acid, tannic acid has been used as standard to confirm by comparing the peaks of chemical compounds from crude extract of *L. flavescens* with retention time of standard. Standard of quarcetin, gallic acid, tannic acid were also separated by using HPLC. From Figure

4.5, Figure 4.6, Figure 4.7, the quarcetin sample showed one of the highest peaks at retention time of 3.936, the gallic acid sample showed one of the highest peaks at retention time of 2.604, the tannic acid sample showed one of the highest peaks at retention time of 2.267 respectively, and it indicated that the sample was pure and not contaminated with other compounds. From this results flavonoid and phenol compounds has been detected from leaves and stems crude extract of *Leptospermum flavescens*. Flavonoids are a large group of polyphenolic compounds that are naturally occurring in plants (Manach, *et al.*, 2004).

The LCMS/MS is approach enormously increased detection sensitivity by over 1000 times. The method developed is also straightforward and convenient and requires no expensive tandem equipment. In this study LCMS/MS analyses were performed using full scan with MS/MS data collection.

The detection of compounds from *Leptospermum flavescens* was analyzed by the present LCMS/MS method. Figure 4.8 – Figure 4.20 shows some representative ion chromatograms (RICs). Consistent with the general findings, 13 labeled compounds were detected as aromadendrin glucoside, hydroxybenzyl hexose, hyperin, kaempferol rhamnoside, quercetin or herbacetin rhamnoside, narigenin, quinic acid, vindoline, flavonol glycosides, protocatechuic acid, methyl epicatechin gallate, apigenin and tetrahydroxy chalcone from leaves and stems crude extract of *L. flavescens* (Table 4.20). From the detection of compounds by LCMS/MS have been analyzed the leaves water crude extract of *L. flavescens* found more active compounds comparing to other crude extract of *L. flavescens*. In comparison with the commonly used thin-layer chromatography (TLC) methods, the LCMS/MS method showed much superior quality in terms of sensitivity and specificity in the detection of compounds.

Although a large number of compounds have been isolated from leaves and stems of *L. flavescens*, few of them have been studies for antidiabetic activity. Some of these compounds were found to reverse the damage to the beta cells and actually repopulate the islets, causing a nearly complete restoration of normal insulin secretion (Li, *et al.*, 2004). These results suggest that *L. flavescens* has a potential antidiabetic activity. Further experiment of the structure of these compounds are necessary to find out the extract possesses antidiabetic activity to elucidate on the basis of their NMR spectroscopy in combination with UV, IR, and MS spectra data.

Standard curve of gallic acid was developed to determine the total phenolic contents of each crude extract from the leaves and stems of *L. flavescens*. Figure 4.21 showed the standard curve of gallic acid and the equation was used to determine the total phenol content of each extract. From Table 4.15, the leaves water extract of *L. flavescens* showed the highest phenolic content, 163.61 GAE mg/g dry mass and stems hexane crude extract exhibited the lowest phenolic content, 17.85 GAE mg/g dry mass.

Standard curve of quarcetin was developed to determine the total flavonoid contents of each crude extract from the leaves and stems of *L. flavescens*. Figure 4.22 showed the standard curve of quarcetin and the equation was used to determine the total flavonoid content of each extract. From Table 4.17, the leaves hexane extract of *L. flavescens* showed the highest flavonoid content, 145.55 QE mg/g dry mass and stems chloroform crude extract exhibited the lowest flavonoid content, 97.87 QE mg/g dry mass. This test showed the leaves water crude extract of *L. flavescens* exhibited the highest phenolic content and the leaves hexane crude extract of *L. flavescens* exhibited the highest flavonoid content.

BSLA test was used to determine the toxicity of the plant extract in different concentrations. The LC_{50} value of the brine shrimp assay was obtained from the crude extract of hexane, chloroform and water in *L. flavescens*. BSLA results presented by Table 8.4 showed that the stems chloroform extract of *L. flavescens* was non-toxic to the brine shrimp. They exhibited very low toxicity, gave LC_{50} values of 609.14 µg/ml. The leaves chloroform extract of *L. flavescens* was the most toxic (Table 8.3), with LC50 value of 21.54µg/ml. Leaves hexane extract, stems hexane extract, leaves water extract and stems water extract of *L. flavescens* exhibited LC50 value of 124.23 µg/ml, 316.33 µg/ml, 124.57 µg/ml and 295.65 µg/ml respectively. The leaves and stems water extract of *L. flavescens* were the most active in antidiabetic activity and glycogen antidiabetic inhibition. They were exhibited low toxicity on brine shrimp, so it is suggested that the leaves and stems water extract of *L. flavescens* more suitable to be used in medical treatment. No research had been reported to determine the toxicity values of extract from *L. flavescens* by using brine shrimp lethality assay (BSLA).

The hypoglycemic effect of *L. flavescens* used as antidiabetic remedies has been confirmed, and the mechanisms of hypoglycemic activity of these plants are being studied. Glycogen phosphorylase (GP) an activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate. And then the phosphate absorbance was measured at 655 nm. Caffeine was used as the standard in the glycogen antidiabetic inhibitory assay. The ability of the Caffeine acted as GP inhibitor to inhibit the process of glycogen degradation and reduce hepatic glucose production (HGP). Various concentration of Caffeine were tested and showed at Table 4.20. At the concentration of 0.6 mg/ml, the percentage of GP inhibitor was 97 %. The GP inhibitory activities of hexane, chloroform and water extracts from the leaves and

stems of *L. flavescens* were determined (Table 4.20). All the crude extract with 5 different concentrations was also tested by using GP inhibitory activity to measure the percentage inhibition of GP activity. The percentage of GP inhibition increased when the concentration of test samples increased. Figure 4.23 showed the GP inhibition of leaves and stems extract of *L. flavescens* and Caffeine. The glycogen antidiabetic (GP) inhibition of crude extract by *L. flavescens* has the potential to be an effective therapeutic strategy for the treatment of type 2 diabetes, as evidenced by studies showing the high percentage inhibition of GP especially in leaves crude extract. The BSLA test also showed the water extract of *L. flavescens* have been exhibited low toxicity.

In animal study, leaves water extract and stems water extract of *L. flavescens* were used to comparing the potential as antidiabetic agent in Alloxan induced rats. When *L. flavescens* water extracts were administered to glucose loaded normal rats (OGTT) fasted for 18 h, reduction in blood glucose levels was observed after 60 minutes. The decline reached its maximum at 2 h (Figure 4.24). Acute toxicity study revealed the non-toxic nature of the extracts. There was no lethality or any toxic reactions found at any of the doses selected until the end of the study period. The water extract of *Leptospermum flavescens* did not show any mortality and none of the treated rats showed any visible symptoms of toxicity up to a dose of 0.5g/kg body weight. Even at this high dose these was no gross behavioral changes indicating high margins of safety (Table 8.15 – Table 8.20). The present study is the preliminary assessment of the antidiabetic activity of the water extracts of *L. flavescens*. The extracts showed a dose-dependent fall in FBGL in Alloxan induced diabetic rats. Alloxan induces diabetes by pancreatic cell damage mediated through generation of cytotoxic oxygen free radicals. The primary target of these radicals is the DNA of pancreatic cells causing DNA

fragmentation (Shankar, *et al.*, 2007). In this study, the difference observed between the initial and final fasting blood glucose levels of different groups under investigation revealed an elevation in blood glucose in diabetic control group at the end of the 20-day experimental period. Administration of water extracts of *L. flavescens* to diabetic rats showed a decrease in the fasting blood glucose level. After 20 days of treatment with *L. flavescens* water extract, maximum reduction in plasma blood glucose level was observed in diabetic rats treated with leaves from *L. flavescens* dose at 0.5 g/kg body weight, with leaves from *L. flavescens* dose at 0.2 g/kg body weight, with stems from *L. flavescens* dose at 0.5 g/kg body weight and with stems from *L. flavescens* dose at 0.2 g/kg body weight. The FBGL became normal by day 20^{th} (Table 4.21). The percentage of GP inhibition in *L. flavescens* increased when the concentration of test samples increased also showed by an elevation of dose in crude extract of *L. flavescens* can be the positive effect on fasting blood glucose level FBGL, serum lipid profile, liver function and changes in body weight (BW). The crude extract of *L. flavescens* also have high percentage of GP inhibition as evidenced by animal studies showing the potential to be an effective therapeutic strategy for treatment of type II diabetes such decreased the FBGL, serum lipid profile, liver function and changes in body weight (BW) became normal by day 20^{th}. The separation of chemical compound from the leaves and stems crude extract of *L. flavescens* were done by TLC showed the presence of alkaloid, flavonoid, saponin, terpenoid, and essential oil. Further analysis by LCMS/MS labeled compounds were detected as methyl epicatechin gallate has hypoglycemic effect due to regeneration of β-cells in the pancreatic islets of Alloxan diabetic rats (Perez, *et al.*, 1998), quercetin or herbacetin rhamnoside that stimulatory compounds such at least in part, exert their effects on insulin release via changes in Ca^{2+} metabolism (Perez, *et al.*, 1998), kaempferol rharnnoside that flavonoids that produced hypoglycemia in rabbits (Perez, *et al.*, 1998), vindoline showed the hypoglycemic activity was observed by

administered orally in a dose of 100 mg/kg (Perez, *et al.*, 1998), aromadendrin glucoside, hydroxybenzyl hexose, hyperin (Li, *et al.*, 2004), narigenin (Fernandes, *et al.*, 2009), quinic acid, flavonol glycosides, protocatechuic acid, apigenin and tetrahydroxy chalcone from leaves and stems crude extract of *L. flavescens* (Table 4.20) showed antidiabetic potential that have been positive effect on fasting blood glucose level, serum lipid profile, liver function and changes in body weight became normal by day 20^{th}. . Most of these compounds showed a mechanism to improve the function of β-cells of pancreatic islets(Li, *et al.*, 2004). This study showed the leaves crude extract of *L. flavescens* exhibited the highest antidiabetic compounds especially in leaves water crude extract comparing to stems water crude extract.

CHAPTER 6

6.0 CONCLUSIONS

TLC analysis exhibited leaves and stems from hexane, chloroform and water of *L. flavescens* have a few of chemical compounds while flavonoid and phenolic compounds were found in this plant by HPLC analysis but HPLC chromatograms were poorly separated. LCMS/MS have been done to detect the specific compounds in this plant. From the previous study, it reported that some of these compounds in *L. flavescens* that detected as antidiabetic agents. However this analysis compounds need the further analysis of structure these compounds to find out the extract possesses antidiabetic activity. The total phenol and flavonoid test showed the leaves of *L. flavescens* exhibited the highest phenol and flavonoid content. This plant exhibited non-toxicity and can be used to animal test for observation of effect on fasting blood glucose level, serum lipid profile, liver function and changes in body weight exhibited some of chemical compounds of *L. flavescens* can be as antidiabetic agent for NIDDM treatment. Selection of the water extract of *L. flavescens* given orally for a period of 20 days produced a decreased plasma glucose level and it does not appear to be toxic, the plant has a hypoglycacmic effect and may be safe when taken orally if the results obtained with rats are applicable to man.

This study showed that *L. flavescens* can be as an antidiabetic medicinal plant. Hence, it might help in preventing diabetic complications and may serve as a good alternative in the present armamentarium of antidiabetic drugs. In addition to the proper utilization of technological advances, a logical interpretation of the codified language of traditional medicine also becomes a necessity in order to further promote to drug-development programmed should be undertaken to develop modern drugs with the compounds

isolated from *L. flavescens*. Although crude extracts from various parts of *L. flavescens* have medicinal applications from time immemorial, modern drugs can be developed after extensive investigation of its bioactivity, mechanism of action, pharmaco therapeutics, and toxicity and after proper standardization and clinical trials. As the global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, development of modern drugs from *L. flavescens* should be emphasized for the control of various diseases especially in diabetes diseases. In fact, time has come to make good use of centuries old knowledge on *L. flavescens* through modern approaches of drug development. Several therapeutically and industrially useful preparations and compounds have also been marketed, which generates enough encouragement among the scientists in exploring more information about this medicinal plant. An extensive research and development work should be undertaken on *L. flavescens* and its products for their better economic and therapeutic utilization.