

ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES, SCHOOL OF MEDICINE, DEPARTMENT OF PHARMACOLOGY

HYPOGLYCEMIC AND ANTIHYPERGLYCEMIC EFFECT OF LEAVES EXTRACTS OF *PSIDIUM GUAJAVA* IN NORMOGLYCEMIC AND STREPTOZOTOCIN-INDUCED DIABETIC MICE

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June, 2015

Addis Ababa, Ethiopia

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A THESIS SUBMITTED TO THE DEPARTMENT OF PHARMACOLOGY, SCHOOL OF MEDICINE, COLLEGE OF HEALTH SCIENCES, ADDIS ABABA UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL PHARMACOLOGY

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School of Graduate Studies

This is to certify that the thesis prepared by Mr. Amanuel Godebo entitled: *Hypoglycemic and antihyperglycemic effect of leaves extracts of Psidium guajava in normoglycemic and streptozotocin-induced diabetic mice* and submitted in partial fulfillment of the requirements for the degree of Master of Science in Medical Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Table of Contents

Acknowledgements I

Table of Contents II

List of Tables IV

List of Figures IV

List of Abbreviations V

Abstract VI

1. Introduction 1

1.1. Diabetes Mellitus 1

1.1.1. Prevalence of Diabetes Mellitus 1

1.1.2. Types of Diabetes Mellitus 1

1.1.3. Diagnosis of Diabetes Mellitus 3

1.1.4. Pathophysiology of Type 2 Diabetes Mellitus 4

1.1.5. Treatment and Management of Type 2 Diabetes Mellitus 4

1.2. Medicinal Plants 5

1.2.1. Role of Medicinal Plants in Diabetic Mellitus 6

1.2.2. Medicinal Effects of *Psidium guajava* 6

1.2.3. Hypoglycemic and Antihyperglycemic effect of *Psidium guajava* 8

1.3. Hypothesis 9

2. Objectives 10

2.1. General objectives 10

2.2. Specific Objectives 10

3. Methods and Materials 11

3.1. Collections and preparation of plant materials 11

3.2. Chemicals and Instruments 11

3.3. Extraction 11
3.4. Animal Preparation 12
3.5. Administration of extract to normoglycemic mice 12
3.6. Preparation of diabetic mice 12
3.7. Administration of extract to streptozotocin-induced diabetic mice 13
3.8. Phytochemical analysis of *Psidium guajava* leaf extracts 13
3.8.1. Detection for alkaloids 13
3.8.2. Detection for Phenols 13
3.8.3. Detection for Flavonoids 14
3.8.4. Detection for Tannins 14
3.8.5. Detection for Saponins 14
3.9. Acute toxicity studies 14
3.10. Ethical considerations 14
3.11. Statistical analysis 15
4. Result 16
4.1. Phytochemical Composition of *Psidium guajava* leaf extracts 16
4.2. Hypoglycemic effect of aqueous and ethanolic extract of *Psidium guajava* 16
4.3. Antihyperglycemic effect of aqueous and ethanolic extract of *Psidium guajava* 17
4.4. Acute toxicity of Aqueous and Ethanolic Extracts of *Psidium guajava* 17
5. Discussion 21
6. Conclusion and Recommendation 24
6.1. Conclusion 24
6.2. Recommendation 24
References 25

List of Tables

Table 1: Classification of oral anti-diabetic agents..... 4

Table 2: Phytochemical screening of aqueous and ethanolic extract of *Psidium guajava* 16

Table 3: Acute oral toxicity of aqueous and ethanolic extract of *Psidium guajava* 18

Table 4: Hypoglycemic effect of aqueous and ethanolic extracts of *Psidium guajava* in normoglycemic mice..... 19

Table 5: Antihyperglycemic effect of aqueous and ethanolic extract of *Psidium guajava* in Streptozotocin-induced diabetic mice..... 20

List of Figures

Figure 1: Spectrum of glucose homeostasis and diabetes mellitus. FPG - Fasting Plasma Glucose; PG - Plasma Glucose. 3

Figure 2: Essential elements in comprehensive diabetes care of type 2 diabetes. 5

Figure 3: *Psidium guajava* (guava) leaf..... 6

List of Abbreviations/Acronyms

DM:	Diabetes Mellitus
FPG:	Fasting Plasma Glucose
IDF:	International Diabetes Federation
GDM:	Gestational Diabetes Mellitus
OECD:	Organisation for Economic Cooperation and Development
OGTT:	Oral Glucose Tolerance Test
STZ :	Streptozotocin/Streptozocin
T1DM :	Type 1 Diabetes Mellitus
T2DM:	Type 2 Diabetes Mellitus
USD:	United States Dollar
WHO:	World Health Organization

Abstract

Background: Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia. As the morbidity and mortality rate from diabetes is increasing in low- and middle-income countries, it is necessary to evaluate medicinal plants for their pharmacological effects to support existing drugs in treating diabetes. The present study was undertaken to evaluate the hypoglycemic and antihyperglycemic activity of leaf extracts of *Psidium guajava* in normoglycemic and streptozotocin-induced diabetic mice.

Methods: The aqueous and ethanol extracts of *Psidium guajava* leaves were prepared. Swiss albino mice of either sex weighing 20-30 grams were selected for the experiments. Normal mice were grouped into eight groups to carry out hypoglycemic effect of the extracts, whereas mice that were made diabetic were grouped into nine groups to study the antihyperglycemic effect of the extracts. Diabetes was induced by Streprozotocin (STZ). Blood glucose levels were measured using glucose oxidase method.

Results: The aqueous and ethanol extracts of *Psidium guajava* leaves lack hypoglycemic effect on normoglycemic mice. However, a significant decrease in blood glucose levels ($P<0.05$) was observed after administering for 21 days treatment of the aqueous and ethanolic extracts of *Psidium guajava* leaf to Streptozotocin-induced diabetic mice. Guava leaf extracts contain alkaloids, phenols, flavonoids, tannins and saponins that could be responsible for the observed antihyperglycemic effect. It was also observed that the extracts have shown no acute toxicity.

Conclusion: The aqueous and ethanol extracts of *Psidium guajava* leaves are effective in lowering blood glucose level in diabetic mice, but lack hypoglycemic effect in normoglycemic mice. Further studies are warranted to elucidate possible mechanism(s) of action of the plant material.

Key words: Hypoglycemic, Antihyperglycemic, *Psidium guajava*, Strepotozotocin, Mice.

1. Introduction

1.1. Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of DM include long term damage, dysfunction and failure of various organs. DM may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. In its most severe forms, ketoacidosis or a non–ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death (WHO, 1999).

The human and economic costs of epidemic of diabetes are enormous. Mortality from communicable diseases and infant and maternal mortality in less-developed countries are declining. In association with increasing diabetes prevalence, this will inevitably result in increasing proportions of deaths from cardiovascular disease in these countries, as well as increased prevalence and associated consequences of other complications of diabetes. A concerted, global initiative is required to address the diabetes epidemic (Wild *et al.,* 2004). Moreover, at least three interventions for prevention and management of diabetes are shown to reduce costs while improving health. Blood pressure and glycemic control, and foot care are feasible and cost-effective interventions for people with diabetes, including in low- and middle income countries (WHO, 2011).

1.1.1. Prevalence of Diabetes Mellitus

According to the report of International Diabetes Federation (IDF), more than 371 million people have diabetes worldwide. Four out of five people with diabetes live in low- and middle-income countries and half of the people who died from diabetes were under the age of 60. Besides, 4.8 million people died and 471 billion USD were spent due to diabetes in 2012. Africa has the highest mortality rate due to diabetes compared to other parts of the world (IDF, 2012).

1.1.2. Types of Diabetes Mellitus

The three main types of diabetes – type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes –occur when the body cannot produce enough of the hormone

insulin or cannot use insulin effectively. People with T1DM, the result of an autoimmune process with very sudden onset, need insulin therapy to survive. T2DM, on the other hand, can go unnoticed and undiagnosed for many years. In such cases, those affected are unaware of the long-term damage being caused by their disease. Gestational diabetes mellitus (GDM), which appears during pregnancy, can lead to serious health risks to the mother and her infant and increase the risk for developing T2DM later in life (IDF, 2013).

T1DM was previously called insulin dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. Although disease onset can occur at any age, the peak age for diagnosis is in the mid-teens. T1DM develops when the cells that produce the hormone insulin, known as the beta cells, in the pancreas are destroyed. This destruction is initiated or mediated by the body's immune system and limits or completely eliminates the production and secretion of insulin, the hormone that is required to lower blood glucose levels. To survive, people with T1DM must have insulin delivered by injection or a pump (CDC, 2014).

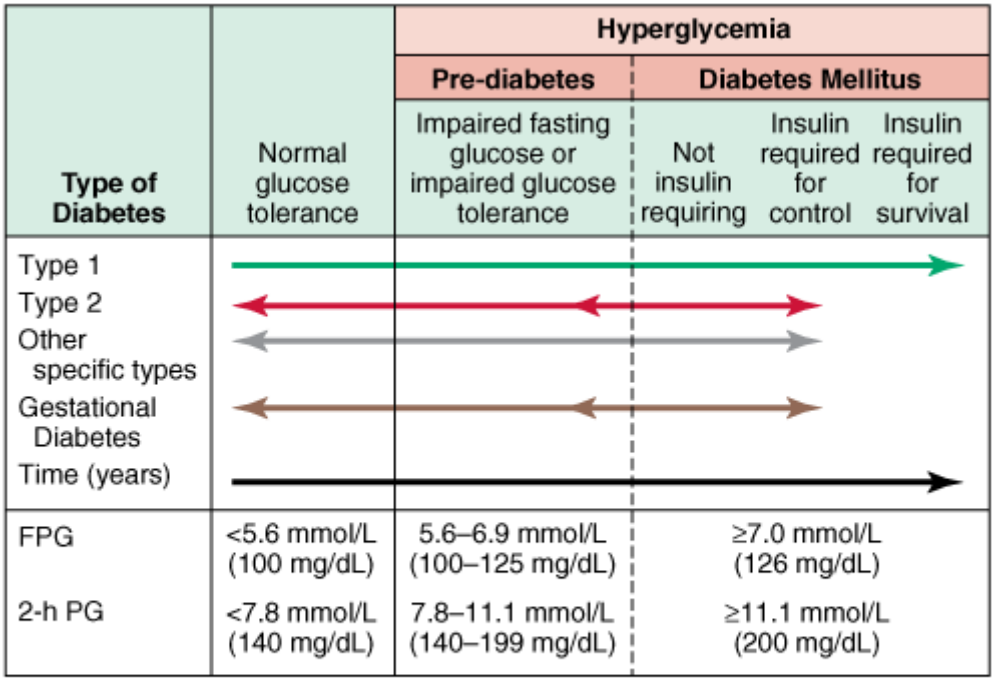
T2DM is the most common form of the disease, accounting for about 90 to 95 percent of diagnosed diabetes cases in U.S. adults. The risk for developing T2DM is associated with older age, obesity, family history of diabetes, history of gestational diabetes, impaired glucose metabolism, physical inactivity, and race/ethnicity (Cerio, 2015). Appropriate medication management targeting glycemic control, hypertension, and lipid management is important for reducing morbidity and mortality, and improving long-term quality of life for patients diagnosed with T2DM. Lifestyle changes such as nutrition therapy, weight loss, increased exercise, and appropriate education and self-management strategies are pivotal to improved outcomes. Inadequate access to care for chronic disease management as well as the cost of medication can contribute to poor control of T2DM and associated cardiovascular risk factors (Redmon *et al.*, 2014).

GDM was defined as decreased carbohydrate tolerance that develops or is first identified during pregnancy, but in 2010 the definition was changed as follows: GDM is a carbohydrate intolerance that is not diabetes that has developed or been discovered for the first time during pregnancy. The GDM definition therefore does not include overt diabetes in pregnancy. Accordingly, hyperglycemic disorders that are thought to have been overlooked until the pregnancy are excluded from the definition of GDM and are instead diagnosed as "overt diabetes

in pregnancy." (Sugiyama 2011) Approximately 7% of all pregnancies (ranging from 1 to 14%, depending on the population studied and the diagnostic tests employed) are complicated by GDM, resulting in more than 200,000 cases annually from 2008-2009 (American Diabetes Association, 2012).

1.1.3. Diagnosis of Diabetes Mellitus

As shown in figure 1 below (Fauci *et al.*, 2008), if the fasting plasma glucose (FPG), i.e. no caloric intake for at least 8 hours, is greater or equal to 7.0mmol/L (126mg/dl) in the case of symptomatic hyperglycemia, the diabetes is confirmed. In case of random plasma glucose or 2h – plasma glucose in 75g oral glucose tolerance test (OGTT), if the plasma glucose level is greater or equal to 11.1mmol/L (200mg/dl), diabetes is confirmed. In the absence of symptomatic hyperglycemia, a repeat confirmatory laboratory test must be done on another day (Ronald Goldenberg *et al.*, 2013).



Source: Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine*, 17th Edition: http://www.accessmedicine.com

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Figure 1: Spectrum of glucose homeostasis and diabetes mellitus. FPG - Fasting Plasma Glucose; PG - Plasma Glucose.

1.1.4. Pathophysiology of Type 2 Diabetes Mellitus

The pathophysiology of T2DM is multi-faceted and includes deficient insulin secretion from pancreatic islet cells, insulin resistance in peripheral tissues, and inadequate suppression of glucagon production. These processes result in inadequate uptake, storage, and disposal of ingested glucose accompanied by elevated hepatic glucose production and hyperglycemia (Boada & Martínez-Moreno, 2013). Impaired insulin secretion is generally progressive, and its progression involves glucose toxicity and lipo-toxicity. The progression of the impairment of pancreatic cell function greatly affects the long-term control of blood glucose. Whereas insulin resistance is a condition in which insulin in the body does not exert sufficient action proportional to its blood concentration. The impairment of insulin action in major target organs such as liver and muscles is a common pathophysiological feature of T2DM (Kaku, 2010).

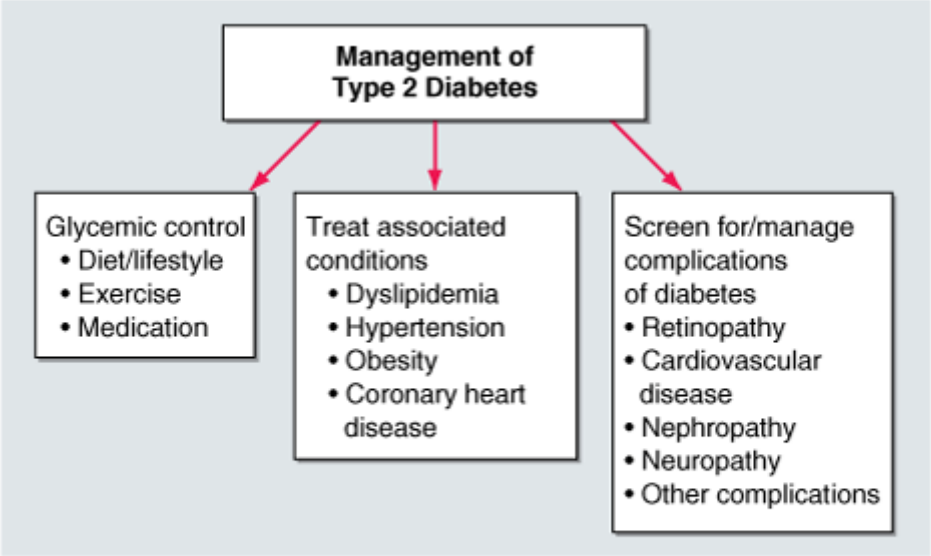
1.1.5. Treatment and Management of Type 2 Diabetes Mellitus

The goal of diabetes treatment is to secure a quality of life and lifespan comparable to those of healthy people, and a prerequisite for attaining this goal is the prevention of onset and progression of vascular complications (Kaku, 2010).

Table 1: Classification of oral anti-diabetic agents

<table>
<tr><td>Sulfonylureas</td><td>Biguanides</td><td>Alpha glucosidase inhibitors</td></tr>
<tr><td rowspan="8">1st generation
• Acetohexamide
• Tolbutamide
• Tolazamide
• Chlorpropamide
2nd generation
• Glibenclamide
• Gliclazide
• Glimepiride
• Glipizide</td><td>• Buformin
• Metformin
• Phenformin</td><td>• Acarbose
• Miglitol
• Voglibose</td></tr>
<tr><td>Meglitinides</td><td>Aldose reductase inhibitors</td></tr>
<tr><td>• Nateglinide
• Repaglinide</td><td>• Epalrestat
• Sorbinil</td></tr>
<tr><td>Thiazolidinediones</td><td>Miscellaneous</td></tr>
<tr><td>• Pioglitazone
• Rosiglitazone
• Troglitazone</td><td>• Glybuzole
• Glymidine
• Midaglizole</td></tr>
</table>

Pharmacological interventions in the treatment of T2DM include insulin therapy, oral hypoglycemic agents (see table 1 above) such as sulfonylureas, biguanides, thiazolidinediones, aldose reductase and alpha-glucosidase inhibitors; and herbal treatment modalities (Bastaki, 2005).



Source: Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine*, 17th Edition: http://www.accessmedicine.com
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Figure 2: Essential elements in comprehensive diabetes care of type 2 diabetes.

1.2. Medicinal Plants

The term medicinal plant includes various types of plants used in herbalism. Some of these plants have proven medicinal activities. Medicinal plants are considered a rich source of ingredients that can be used in drug development and synthesis. Besides these plants play a critical role in the development of human cultures around the world (Hassan, 2012). At the present moment, phytochemistry and pharmacological analysis of traditional medicines occupy a key position in medicinal plant research and indigenous knowledge systems. Sharing of such knowledge is crucial for maintaining options for the use of traditional medicines, particularly as use of alternative medicine is growing because of its moderate costs and increasing faith in herbal medicines by the public (Maroyi, 2013).

1.2.1. Role of Medicinal Plants in Diabetic Mellitus

For diabetes mellitus, therapies developed along the principles of western medicine (allopathic) are often limited in efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world. Therefore, treating diabetes mellitus with plant-derived compounds which are accessible and do not require laborious pharmaceutical synthesis seems highly attractive (Rao *et al.,* 2010).

1.2.2. Medicinal Effects of *Psidium guajava*

Psidium Guajava is called Zeytuna ("ዘይቱና") in Ethiopia, guayaba in Spanish speaking countries and goiaba in Brazil; guava is a common shade tree or shrub in door-yard gardens in the tropics. It belongs to family of Myrtaceae, genus: *Psidium*, species: *guajava* and common names of the plant are guava, goiaba, guayaba etc. (Vyas *et al.*, 2010). It is originated in the tropical South America and grows widly in Bangladesh, India, Thailand, Brazil, Florida, and West Indies, California and also in several other countries (Mittal *et al*., 2010). Generally, guava plant has spread widely throughout the tropics because it thrives in a variety of soils, propagates easily, and bears fruit relatively quickly. The guava berry is an important tropical fruit that is mostly consumed fresh. (Joseph *et al*., 2011).



Figure 3: *Psidium guajava* (guava) leaf

The ethanolic extract of guava (*Psidium guajava* L.) leaves have anti-inflammatory effect. The guava leaf extract significantly inhibited lipopolysaccharide-induced production of inflammatory mediators, such as nitric oxide and prostaglandin E_2 in dose dependent manner. The extract also suppressed induced nitric oxide synthase and cyclooxygenase-2 expression through suppression of the ERK1/2 MAPK signaling pathway. So, it may have a potential to prevent inflammatory disorders (Jang *et al.,* 2014). Moreover, the aqueous extract of *Psidium guajava* Linn. leaves showed hepatoprotective activity when administered at doses 250mg/kg and 500mg/kg orally in carbon tetrachloride (CCl_4) induced acute and chronic liver damage, paracetamol-induced liver damage and thioacetamide-induced liver damage. This activity may be due to an antioxidant effect of the plant (Roy *et al.,* 2006).

In a study done in both rats and guinea pigs, the aqueous extract of Psidium guajava (5g/kg, orally) showed a marked anti-cough activity, although the effect observed was less potent when compared to 3mg/kg dextromethorphan (Jaiarj *et al.,* 1999). The aqueous extract of unripe *Psidium guajava* fruit peel showed significant hypolipidemic, hypoglycemic and antidiabetic activity by lowering their triglyceride, total cholesterol, high density lipoprotein, alkaline phosphatase, aspartate amino transeferase, alanine amino transferase and creatanine levels in streptozotocin-induced diabetic rats. This effect could be beneficial in T2DM (Rai *et al.,* 2010).

Ethanolic extract of *Psidium guajava* leaves exerts its antidiarrheal activity by a dual action of its antimicrobial effect and the reduction of gastrointestinal motility. The numerous tannins, polyphenolic compounds, flavonoids, ellagic acid, triterpenoids, guiajaverin, quercetin, and other chemical compounds present in the plant are speculated to account for the observed effects (Ezekwesili *et al.,* 2010). Antibacterial activity of aqueous and methanolic extract of *Psidium guajava* was observed on *in-vitro* study done against multi drug resistant clinical isolates of *Staphylococcus aureus* strains. This activity was possibly due to protein-degrading activity of the extract (Anas *et al.,* 2008; Gonçalves *et al.,* 2008). Besides, the aqueous extracts of dried leaves and barks of *Psidium guajava* were effective against inhibiting the growth of bacteria: *Staphylococcus aureus,* and *Staphylococcus epidermidis* and fungi: *Microsporum gypseum* and *Trichophyton mentagrophytes* (Richard *et al.,* 2013).

In general, the pharmacological studies conducted on *Psidium guajava* indicate the immense potential of this plant in the treatment of conditions such as diarrhoeal, gastroenteritis and

rotavirus enteritis, wounds, acne, dental plaque, malaria, allergies, coughs, diabetes, cardiovascular disorder, degenerative muscular diseases, inflammatory ailments including rheumatism and menstrual pain, liver diseases, cancer, etc. Not surprisingly, guava also exhibits antioxidant and anti-inflammatory effects as oxidative injury underlies many of these diseases (Gutierrez *et al.,* 2008).

1.2.3. Hypoglycemic and Antihyperglycemic effect of *Psidium guajava*

The butanol-soluble fraction from of *Psidium guajava* leaves possesses beneficial effects on the treatment and prevention of hyperglycemia in type 2 diabetes and these effects are, at least in part, mediated via the inhibition of protein tyrosine phosphatase1B (PTP1B), which is a major mediator of insulin signaling and insulin resistance (Oh *et al.,* 2005). An ethanol extract of the stem bark of *Psidium guajava*, an indigenous medicinal plant used to control diabetes in Indian System of Medicine, exhibited statistically significant hypoglycemic activity in alloxan-induced hyperglycemic rats but was devoid of significant hypoglycemic effect in normal and normal glucose loaded rats (OGTT) (Mukhtar *et al.,* 2006).

The hypoglycemic effect of the leaf extract of *Psidium guajava* Linn. (family, Myrtaceae) was examined in normal and diabetic rats, using streptozotocin (STZ)-induced diabetes mellitus model. Chlorpropamide (CPP; 250 mg/kg, p.o.) was used as the reference hypoglycemic agent for comparison. Acute oral administrations of the plant's extract (PGE; 50-800 mg/kg, p.o.) caused dose-related, significant ($p < 0.05$-0.001) hypoglycemia in normal (normoglycemic) and STZ-treated, diabetic rats. The results of this experimental animal study indicate that the leaf aqueous extract of *Psidium guajava* possesses hypoglycemic, and thus lend pharmacological credence to the suggested folkloric, ethno-medical uses of the plant in the management of adult-onset, type 2 diabetes mellitus in some rural African communities (Ojewole, 2005).

The water-soluble extract from *Psidium guajava Linn* significantly inhibited, in a dose-dependent manner, the activities of alpha-glucosidase from small intestinal mucosa of diabetic mice. The aqueous guava leaf extract inhibition concentration (IC_{50}) to sucrase or maltase was 1.0 g/L or 3.0 g/L respectively (Wang *et al.,* 2007). In other study, a water extract of *Psidium guajava* leaves was screened for hypoglycemic activity on alloxan-induced diabetic rats. In both

acute and sub-acute tests, the water extract, at an oral dose of 250 mg/kg, showed statistically significant hypoglycemic activity (Mukhtar *et al.*, 2004).

In the developing world, the majority of people use traditional medicine to prevent and treat diseases and the medicinal plants stand in the first place for this purpose. Secondly, medicinal plants are a potential source for the development of new drugs to diagnose, prevent and treat diseases. So, it is necessary to evaluate medicinal plants for their pharmacological effects and identify which part of the plant is more appropriate to use for the intended purpose. Moreover, it will provide the basic information about the plant to investigate further.

1.3. Hypothesis

Aqueous and ethanol extracts of *Psidium guajava* leaves have hypoglycemic effect on normoglycemic mice and antihyperglycemic effect on streptozotocin-induced diabetic mice.

2. Objectives

2.1. General objectives

This study is intended to investigate whether or not the aqueous and ethanolic extracts of *Psidium guajava* leaves have hypoglycemic and antihyperglycemic effect in mice.

2.2. Specific Objectives

- To evaluate hypoglycemic effect of aqueous and ethanolic extracts of *Psidium guajava* leaves on normoglycemic mice
- To evaluate antihyperglycemic effect of aqueous and ethanolic extracts of *Psidium guajava* leaves on streptozotocin-induced mice
- To carry out phytochemical screening for alkaloids, phenols, flavonoids, tannins and saponins
- To evaluate the acute toxicity of the leaf extracts of *Psidium guajava*

3. Methods and Materials

3.1. Collections and preparation of plant materials

Fresh leaves of *Psidium guajava* were collected from Wolaitta Zone, South Nation's and Nationalities Peoples Region located about 330 kilometer south of the capital city, Addis Ababa, Ethiopia. After collection, the plant materials were identified and authenticated by Dr. Getachew Addis, a taxonomist, Traditional and Modern Medicine Research Directorate, Ethiopian Public Health Institute, and deposited with a voucher number AG001. The guava leaves were then dried under shade and crushed to powder for extraction.

3.2. Chemicals and Instruments

Streptozotocin (STZ) [S0130 SIGMA-ALDRICH, USA] was obtained from the chemicals and reagents store of the Department of Biochemistry, School of Medicine, College of Health Science, Addis Ababa University. Glibenclamide [Cadila Pharmaceuticals (Ethiopia) PLC], ethanol (WINLAB, Leicestershire, UK), potassium iodide (Fischer Chemical, UK), bismuth (III) nitrate basic (Fluka, Spain), acetic acid glacial (LABORT, India), lead acetate (Nakarai Chemicals ltd., Japan), Picric acid (MERCK, Germany) and Iron (III) chloride (Seelze-Hannover, Germany) were obtained from Traditional and Modern Medicine Research Directorate, Ethiopian Public Health Institute. Other chemicals used were also of analytical grade. Lyophilizer (LANCONCO – Freeze Dry System, USA), Rota vapor (Rotary Evaporator RE300, UK) and water bath (KOTTERMANN, Germany) were used to dry the extracts. Blood glucometer (SensoCard – Blood Glucose Meter, Hungary) was used to measure the glucose concentration in the whole blood of mice. Test strips (SensoCard – Blood glucose test strips, Hungary) were used to detect the glucose level using glucometer by applying enzyme glucose oxidase which is specific for β-D-glucose measurement.

3.3. Extraction

For ethanol extract, the powdered leaves (300g) were extracted by maceration using 70% ethanol and the mixture was filtered using Whatmann filter paper no. 1. The extract was made ethanol free by evaporating it using rotary vaporizers under reduced pressure. The extract was made

solvent free by keeping it in water bath at 40^0c. The yield obtained was 7.5% (w/w). Then, it was kept in refrigerator at 8 degree centigrade and fresh stock solution was prepared for the experiment whenever required. The aqueous extract was prepared by maceration of the powdered leaves (300g) by using distilled water. Subsequently, the mixture was filtered using Whatmann filter paper no. 1. The extract was set water free by using Lyophilazer. The yield obtained was 8.5% (w/w).

3.4. Animal Preparation

Healthy, Swiss albino mice of either sex weighing 20-30 grams were brought from Experimental Animal Breeding Unit, Ethiopian Public Health Institute: the animals were breed using a standard diet and tap water *ad libitum* in an environment of a 12-hour light/dark cycle. Then, the mice were acclimatized prior to use for the study.

3.5. Administration of extract to normoglycemic mice

Mice were divided into eight groups (six test groups and two control groups), each group comprising a minimum of six mice (n=6). The mice were starved overnight prior to performing the experiment. The positive control group received glibenclamide, while the negative control group received distilled water, and the test groups were administered aqueous extracts of 250mg/kg, 500mg/kg and 750mg/kg; and ethanol extract of 250mg/kg, 500mg/kg and 750mg/kg of *Psidium guajava.* The extract was administered orally by using gavage. The effects of the plant extract were compared with the control groups. Blood sample from the control and test animals was collected after 0, 30, 60, 90, 120 and 180 minutes following extract or water administration. Blood glucose level was measured using glucometer on blood drawn from the tail of the mice.

3.6. Preparation of diabetic mice

Mice were fasted for four hours with free access to water, and then injected intraperitonially twice on successive days with streptozotocin dissolved in 2.94% sodium citrate solution (50mg/kg body weight). All animals had free access to water and pellet diet after thirty minutes of administration of Streptozotocin. 72 hours later, blood glucose level were measured in mice fasted for four hours using glucometer; mice with greater than 200mg/dl was defined as DM.

Streptozotocin induced diabetic mice were selected and divided into eight groups; one negative control, one positive control and six test groups.

3.7. Administration of extract to streptozotocin-induced diabetic mice

Streptozotocin-induced diabetic mice were administered (orally) 250mg/kg, 500mg/kg and 750mg/kg of the ethanol and aqueous extracts of *Psidium guajava* for test groups; 0.66mg/kg glibenclamide for positive control group; and distilled water (1ml) for negative control group. There was also normal control group that received distilled water (1ml) orally. Extract, standard drug and water were administered once daily for 21 days. Blood glucose level was measured on 0 day, 7^{th} day, 14^{th} day and 21^{st} day following the induction. The blood glucose level was measured using glucometer blood drawn from the tail of the mice.

3.8. Phytochemical analysis of *Psidium guajava* leaf extracts

Qualitative preliminary phytochemical screening test were carried out for *Psidium guajava* leaf extracts as per the standard methods (Pandey & Tripathi 2014, Herin Sheeba Gracelin et al. 2013, Tiwari et al. 2011) to indicate the presence of alkaloids, phenols, flavonoids, tannins and saponins.

3.8.1. Detection for alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

- ✓ **Dragondorff's test:** Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of yellow orange precipitate indicates the presence of alkaloids.
- ✓ **Hager's test:** Filtrates were treated with Hager's reagent (saturated solution of Picric acid). Presence of alkaloids confirmed by formation of yellow colored precipitate.

3.8.2. Detection for Phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

3.8.3. Detection for Flavonoids

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

3.8.4. Detection for Tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and formation of brownish green or a blue black colouration indicates the presence of tannins.

3.8.5. Detection for Saponins

About 2 g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. If foam produced persists for ten minutes it indicates the presence of saponins.

3.9. Acute toxicity studies

Acute toxicity study was performed on 30 female mice. The animals were kept fasting overnight, but water was provided *ad libitum*. They were divided into six groups, five animals in each group, and the aqueous and alcohol extracts of *Psidium guajava* were administered orally in an increasing dose level of 300, 2000, and 5000mg/kg via oral gavage according to the guidelines of the Organization for Economic cooperation and Development (OECD, 2001). Animals were kept under close observation for 4 hours after administering the extracts for behavioral, neurological and autonomic profile and then were observed for any change in the general behavior and/or other physical activities within 24 hours. Thereafter, the observation continued daily for a total of 14-days.

3.10. Ethical considerations

The experiment was carried out in accordance with ethical principles and the proposal was approved by Addis Ababa University, college of health sciences, faculty of medicine, Department of pharmacology.

3.11. Statistical analysis

The data were expressed as mean ± standard error of mean (SEM). Differences between means of all parameters were carried out by using analysis of variance (ANOVA). Subsequently, the Tukey post-hoc tests with multiple comparisons were used to determine the source of significant differences. $P < 0.05$ was considered to be statistically significant. GraphPad InStat 3 and SPSS Version 20 Software were used for statistical analysis. Percent reduction of blood glucose level was calculated using the following formula:

$\frac{G_0 - G_{21}}{G_0}$, where G_0 is blood glucose level at day 0; G_{21} is blood glucose level at day 21.

4. Results

4.1. Phytochemical Composition of *Psidium guajava* leaf extracts

As the phytochemical screening tests showed in our study, both the aqueous and ethanolic extracts of *Psidium guajava* leaves comprise of many secondary metabolites (see table 2 below). The result revealed that the guava leaf extracts are good sources of alkaloids, phenols, flavonoids, tannins and saponins. The aqueous extract contains higher amount of phenols and saponins than the ethanolic extract, whereas the ethanolic extract contains larger amount of alkaloids and tannins than aqueous extract.

Table 2: Phytochemical screening of aqueous and ethanolic extract of *Psidium guajava*

Chemical Compounds Class	Test Performed	Expected outcome	Result	
			Aqueous Extract	Ethanol Extract
Alkaloids	Dragendorff's Test	Yellow orange precipitate	Positive	Positive
	Hager's Test	Yellow precipitate	Positive	Positive
Phenols	Ferric chloride test	Blue black coloration	Positive	Positive
Flavonoids	Lead acetate test	Yellow color precipitate	Positive	Positive
Tannins	Ferric chloride (0.1%) test	Blue black coloration	Positive	Positive
Saponins	Foam formation test	Foam formation	Positive	Positive

4.2. Hypoglycemic effect of aqueous and ethanolic extract of *Psidium guajava*

After administering the test substance and the standard, the blood glucose levels were measured every 30 minutes for 3 hours post administration. The results are summarized in Table 4. Before initiating the treatment, there were no statistically significant differences in blood glucose level among the whole groups ($P>0.05$). Until it reaches 30min, the result was the same. After 1hr, the blood glucose level of the standard substance showed statistically significant difference from the negative control group and the test groups ($P<0.05$). The negative control group and the whole test groups did not show any significant differences in blood glucose level within 3 hours ($P>0.05$), whereas the blood glucose level of positive control group showed statistically

significant variation after 3 hour of administration (P<0.05). The present results indicate that the aqueous and ethanolic extracts of *Psidium guajava* have no hypoglycaemic effect in normoglycemic mice.

4.3. Antihyperglycemic effect of aqueous and ethanolic extract of *Psidium guajava*

The blood glucose levels of the whole groups were measured once weekly during the three weeks treatment course as shown in Table 5. Before induction of diabetes, the treatment groups had no statistically significant difference in their blood glucose levels (P>0.05). The blood glucose levels were not significantly different between the standard and test groups (P>0.05) before initiating the treatment, except the normal control (non-diabetic) group. The standard group and the test groups that were receiving 500mg/kg (aqueous), 750mg/kg (aqueous) and 750mg/kg (ethanol) showed statistically significant variation in their blood glucose level within the first week treatment (P<0.05). In addition to the above groups, the blood glucose level of the test group that receiving 500mg/kg (ethanolic extract) was significantly different at the end of the second week of treatment course (P<0.05). On day 21st, the test group which was receiving 250mg/kg (aqueous) also showed statistically significant difference in their blood glucose level. On the last day of the treatment course, the percent reduction in blood glucose level was 8.24, 23.41, 28.76, 6.57, 13.85, 21.66 and 31.61 for those groups receiving 250mg/kg (aqueous), 500mg/kg (aqueous), 750mg/kg (aqueous), 250mg/kg (ethanol), 500mg/kg (ethanol), 750mg/kg (ethanol) and glibenclamide (0.66mg/kg), respectively. Across the experimental period, the normal control (non-diabetic) group and the negative control (diabetic) group did not show any significant difference in their blood glucose level (P>0.05). The present study reveals that the aqueous and ethanolic extracts of *Psidium guajava* have anti-hyperglycaemic effect in diabetic-induced mice.

4.4. Acute toxicity of Aqueous and Ethanolic Extracts of *Psidium guajava*

In the present study, signs of toxicity were not observed, i.e. no change in behaviors such as alertness, restlessness, irritability and fearfulness; as well as no change in autonomic activities such as defecation and urination, following the administration of graded doses of both aqueous and ethanol extracts of *Psidium guajava* (up to a dose of 5000mg/kg) within 24 hours, as shown

in Table 3. Besides, there were no delayed deaths within 14-day observation period up to the dose of 5000mg/kg body weight, indicating that the medium lethal dose (LD_{50}) is greater than 5g/kg body weight in mice. These test results indicate that there is very low potential for acute toxicity from ingesting aqueous and ethanol extracts of *Psidium guajava* through orally. (*Delayed death* means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.)

Table 3: Acute oral toxicity of aqueous and ethanolic extract of *Psidium guajava*

Group	No. of mice	*Psidium guajava* leaf extract Dose (mg/kg)	Toxicity observed		Number of deaths
			Within 24 hrs.	After 14 days	
1	5	300mg (Aqueous extract)	No	No	0*
2	5	2000mg (Aqueous extract)	No	No	0*
3	5	5000mg (Aqueous extract)	No	No	0*
4	5	300mg (Ethanol extract)	No	No	0*
5	5	2000mg (Ethanol extract)	No	No	0*
6	5	5000mg (Ethanol extract)	No	No	0*
7	5	1ml of water (control)	No	No	0*

* At $P<0.05$, there is no statistically significant differences among test and control groups.

Table 4: Hypoglycemic effect of aqueous and ethanolic extracts of *Psidium guajava* in normoglycemic mice

Groups	Blood Glucose Level (mg/dl)						Percent Reduction
	0’	30’	60’	90’	120’	180’	
Negative control group (distilled water)	167.0±5.28	195.7±10.71	195.7±8.77	196.2±10.80	194.2±12.33	147.0±10.73	11.98
Positive control group [Glibenclamide (0.66mg/kg)]	178.0±3.28	166.7±6.84	128.8±7.48*	113.8±7.11*	113.7±3.46*	113.3±10.91*	36.35
Aqueous extract of 250mg/kg	185.0±5.39	195.5±5.55	192.0±4.81	186.3±4.90	169.8±6.75	161.5±2.98	12.70
Aqueous extract 0f 500mg/kg	169.2±4.40	195.3±6.25	183.2±6.37	162.8±7.07	157.5±4.44	171.0±12.38	-1.06
Aqueous extract of 750mg/kg	174.5±3.17	185.8±4.85	179.5±6.75	197.8±7.85	181.2±7.45	166.8±6.25	4.41
Ethanol extract of 250mg/kg	173.5±10.45	182.2±5.97	179.7±7.44	192.0±10.90	176.1±8.65	161.8±9.87	6.74
Ethanol extract of 500mg/kg	187.3±10.94	204.0±18.38	194.8±16.09	197.5±16.80	187.0±20.73	171.7±16.30	8.33
Ethanol extract of 750mg/kg	185.8±11.25	200.5±9.42	191.2±11.47	180.8±9.60	167.8±9.24	174.2±6.61	6.24

*P<0.05, Mean ± Standard Error of Mean, n=6

Table 5: Antihyperglycemic effect of aqueous and ethanolic extract of *Psidium guajava* in Streptozotocin-induced diabetic mice

Groups	Blood Glucose Level (mg/dl)				Percent Reduction
	Day 0	Day 7	Day 14	Day 21	
Normal control group (without streptozotocin)	129.33±3.20	134.50±2.89	135.33±1.94	136.17±1.94	-5.23
Negative control group (diabetic mice)	275.80±6.90	280.40±5.51	280.80±4.37	276.80±5.45	-0.36
Positive control group [Glibenclamide (0.66mg/kg)]	263.20±5.94	228.00±5.61***	205.60±4.85***	180.00±6.59***	31.61
Aqueous extract of 250mg/kg	267.00±12.29	262.33±9.97	250.17±10.12	245.00±7.98*	8.24
Aqueous extract 0f 500mg/kg	272.67±9.05	246.00±8.56*	230.17±9.08***	208.83±7.52***	23.41
Aqueous extract of 750mg/kg	261.33±7.92	230.17±7.88***	209.33±8.11***	186.17±7.50***	28.76
Ethanol extract of 250mg/kg	274.00±7.80	267.67±6.78	260.17±6.45	256.00±5.94	6.57
Ethanol extract of 500mg/kg	257.50±4.55	247.67±5.06	234.00±4.08**	221.83±4.84***	13.85
Ethanol extract of 750mg/kg	249.33±6.53	231.67±6.21**	212.67±5.30***	195.33±5.90***	21.66

*P<0.05, **P<0.01, ***P<0.001 verses negative control (diabetic mice); Mean ± Standard Error of Mean, n=6

5. Discussion

Diabetes is a group of diseases marked by high levels of blood glucose resulting from problems in how insulin is produced, how insulin works, or both. People with diabetes may develop serious complications such as heart disease, stroke, kidney failure, blindness, and premature death (CDC, 2014). In 2002, the estimated cost of treating diabetes and its complications, both in terms of direct (medical) and indirect (disability, work loss, death, etc.) costs, was estimated at $132 billion. The direct medical costs of diabetes more than doubled in five years from $44 billion in 1997 to $92 billion in 2002. Treatment costs for people with diabetes are more than double those for people without diabetes, mainly because of the high costs associated with complications. Cardiovascular disease remains the most costly complication of T2DM (NDEP, 2007). Therefore, people with diabetes need safer and more effective antidiabetic drugs that can decrease diabetic associated chronic complications, especially cardiovascular disorders.

In the present study, the hypoglycemic and antihyperglycemic effects of the aqueous and ethanol extracts of *Psidium guajava* were performed in normoglycemic and streptozotocin-induced diabetic mice, respectively. STZ is one of the most commonly used substances to induce diabetes in rodents. STZ (2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose) is a broad-spectrum antibiotic which is produced by the bacterium *Streptomyces achromogenes* in 1959. The diabetogenic effect of STZ was first reported in 1963 by Rakieten *et al* (Sharad *et al.*, 2010) after injection of a single intravenous dose in rats and dogs. The development of STZ-induced diabetes is associated not only with hyperglycemia, but also with marked necrotic changes in endocrine cells of pancreatic islets, decreased β-islet cell area accompanied by hyperplasia of their nuclei, increased α-endocrine cell area, as well as increased apoptotic index (Smirnov *et al.*, 2012).

The normoglycemic mice were administered a single dose ranging from 250-750mg/kg of aqueous and ethanolic extracts of *Psidium guajava* leaves for each group containing six mice and their blood glucose level were measured every 30minutes until the time reaches 3hours in this study. Both the aqueous and the ethanolic extracts of *Psidium guajava* leaves didn't show any significant hypoglycemic effect after the mice were administered the extracts. Therefore, this study indicates that single dose ranging from 250-750mg/kg of both the aqueous and the ethanolic extracts of *Psidium guajava* leaves have no hypoglycemic effect on normoglycemic mice. The previous study done on normoglycemic mice using an ethanolic extract of the stem bark of *Psidium guajava* was devoid of significant hypoglycemic effect (Mukhtar *et al.*, 2006) which supports our study. However, the acute oral administration of aqueous extract of *Psidium guajava* leaves (dose ranging from 50-800mg/kg) showed hypoglycemic effect (Ojewole, 2005) on normoglycemic mice which has a paradox result with the present study.

After administration of leaf extract of *Psidium guajava* (250mg/kg) to alloxan and STZ-induced diabetic mice, the blood glucose level began to decline within one hour and continued to decline up to 6hr on alloxan-induced diabetic mice; but the blood glucose levels declined to their lowest level after an interval of 4 hours on STZ-induced diabetic mice (Sengupta *et al.*, 2011). The ethanolic extract of *Psidium guajava* leaf has a marked hypoglycemic and hypolipidemic effect on alloxan-induced diabetes (Shakeera *et al.*, 2013). In this study, the aqueous and the ethanolic extract of *Psidium guajava* leaves (dose: 250, 500 and 750mg/kg) were administered daily for three weeks to STZ-induced diabetic mice. The significant reduction of the blood glucose levels of the mice were observed gradually from the first week end to the last week of treatment in the group receiving aqueous extract (250mg/kg, 500 and 750mg/kg) and ethanol extract (500 and 750mg/kg). The present study revealed that both the aqueous and the ethanolic extracts of *Psidium guajava* leaves have a significant antihyperglycemic effect on STZ-induced diabetic mice. The aqueous extract of *Psidium guajava* leaf (750mg/kg) reduced the blood glucose level efficiently by 28.76 percent on 21st day which is almost comparable with the dose of glibenclamide treated group – also showed a significant fall of 31.61 percent.

In the present findings, the leaf extracts of guava showed antihyperglycemic effect on STZ-induced diabetic mice without causing hypoglycemia on normoglycemic mice while glibenclamide showed both the hypoglycemic as well as the antihyperglycemic effect on normoglycemic and STZ-induced diadetic mice. As a result, the mechanism of action of leaf extracts of *Psidium guajava* in the treatment of diabetic mice is unquestionably different from the standard drug glibenclamide, which acts by increasing the insulin secretion from the pancreas and should be used with caution in patients with cardiovascular disease or in elderly patients, in whom hypoglycemia would be especially dangerous (Katzung *et al.*, 2012). However, wide-ranging pharmacological and biochemical researches are required to address the detailed mechanism and active principles responsible for the antihyperglycemic effects observed in the study.

Most of the drugs have definite specific chemical constituents to which their biological or pharmacological activity is attributed. Qualitative and quantitative characterization of the active ingredient should be assayed using biomarkers. Defining of the biomarker has to be very specific and a lot of insight has to go into it before declaring any distinct molecule (Pandey & Tripathi, 2014). Ethanolic leaf extracts of *Psidium guajava* (guava) produced the highest percentage of the phytochemical constituents than the aqueous leaf extracts. Tannins, alkaloids, total polyphenols, saponins and oxalate found in both extracts may be responsible for the folkore and scientifically documented medicinally beneficial effects of the plants (Ojezele & Agunbiade, 2013). Phytochemical screening analysis done on leaves of *Psidium guajava* revealed the presence of flavonoids, tannins, reducing sugars, terpenes, saponins, anthraquinones and alkaloids. The chemical constituents present in the extracts have some therapeutic values (Egga *et al.*, 2014). In our study also, the phytochemical screening supported the previous studies and one of these secondary metabolites may be responsible for their antidiabetic effects.

Acute oral toxicity of the aqueous and ethanol extracts of *Psidium guajva* leaves was also evaluated in this study. And, the extracts found to be relatively safe on acute exposure up to a single oral administration of 5000mg/kg. No sign of toxicity and mortality associated with the aqueous and ethanol extracts of the plant.

6. Conclusion and Recommendation

6.1. Conclusion

In conclusion, this study revealed that the aqueous and ethanolic extracts of *Psidium guajava* leaves are effective in lowering blood glucose level on diabetic mice, but lack hypoglycemic effect on normoglycemic mice. The phytochemical screening indicated the presence of alkaloids, phenols, flavonoids, tannins and saponins. The LD_{50} value of the extract is greater than 5,000 mg/kg which indicates the plant material has a wider safety margin in animal models. Therefore, *Psidium guajava* leaves can be used as an alternative herbal remedy for the treatment of diabetes.

6.2. Recommendation

In future studies, it is important to undergo fractionation and purification using different advanced technologies like HPLC to isolate and identify the exact molecules which are responsible for antidiabetic effect.

The medicinal values of *Psidium guajava* should be given further attention with special reference to its antidiabetic activities.

It is also necessary to perform comparison studies that can designate the antidiabetic activity of guava leaf extracts with the other existing hypoglycemic agents.

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