EFFECT OF *Zanthoxylum armatum* FRUITS METHANOLIC EXTRACT ON SELECTIVE BLOOD METABOLITES AND HEPATIC GLYCOGEN IN NEONATAL STREPTOZOTOCIN INDUCED TYPE-2 DIABETIC RATS

A Thesis
Submitted to
Khulna University, Khulna
In Partial Fulfillment of the Requirements for the Degree of
Master of Science in
Biotechnology and Genetic Engineering
By

Student ID: MS- 180712
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Approved as to the style and content by

Dr. Khondoker Moazzem Hossain
Supervisor
Professor
Biotechnology and Genetic Engineering-Discipline
Khulna University
Khulna-9208, Bangladesh

Dr. Begum Rokeya
Co-Supervisor
Professor & Head
Department of Pharmacology
Bangladesh University of Health Sciences
Mirpur-01, Dhaka-1216, Bangladesh

Dr. Ayesha Ashraf
Chairman, Examination Committee
and
Head, Biotechnology and Genetic Engineering Discipline
Khulna University
Khulna-9208

March, 2020

DECLARATION

This thesis/report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor.

Md. Shamim Gazi

Md. Shamim Gazi
Student ID: MS-180712
March, 2020

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The Author

Abstract

Zanthoxylum armatum locally named as 'Gaira' is used in traditional medicine for the treatment of different ailments including diabetes. The present study was aimed to screen the effect of *Zanthoxylum armatum* fruits methanolic extract (ZAFME) on blood glucose, lipid profile, liver glycogen and enzymes in neonatal streptozotocin (STZ) induced type-2 diabetic rats. STZ was administered in 48 hours old pups by a single intraperitoneal injection and after three months, oral glucose tolerance test was performed. Rats were divided into five groups viz. Standard, Negative Control, Positive Control, Treatment Group 1 and Treatment Group 2. During the 28 days experimental period, the experimental rats were given basal diet as well as Standard and Negative Control groups were provided with pure drinking water, Positive Control group, Treatment Group 1 and Treatment Group 2 were provided with orally Gliclazide, 25 mg and 50 mg/kg body weight of ZAFME, respectively. Blood samples were collected from tail cut at day 1 and by cardiac puncture at day 28. Fasting serum glucose, lipid profile, liver glycogen, SGPT and SGOT were measured. Statistical analysis was performed by one-way ANOVA and paired sample t-test. Blood glucose level was significantly ($p<0.05$) lowered in Treatment Group 1, Treatment Group 2 and Positive Control group at day 28 when compared to day 1. Blood glucose level was significantly ($p<0.05$) decreased in Treatment Group 2 compared to Negative Control group. The level of Triglycerides was reduced significantly ($p<0.05$) in Treatment Group 1 and 2 at day 28 compared to day 1. Total cholesterol and higher HDL levels was lowered in Positive Control and Treatment Group 2. Treatment Group 1 and 2 and Positive Control groups showed lower LDL levels at day 28 compared to day 1. Hepatic glycogen level was increased in Treatment Group 1 and Treatment Group 2 compared to Negative Control group. Treatment Group 1 showed significant ($p<0.05$) reduction of SGPT levels when compared to Negative Control group. However, both Treatment Group 1 and Treatment Group 2 showed significant ($p<0.05$) reduced level of SGOT when compared to Negative Control group. Therefore, it may be concluded that, ZAFME possesses potential antidiabetic properties. The findings highlight the therapeutic potential use of the ZAFME on type 2 diabetes and provide strong impetus for further research studies regarding clinical trials and drug development.

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ABBREVIATIONS

ANOVA	: Analysis of variance
bw	: Body weight
SD	: Standard Deviation
%	: Percent
µg	: Microgram
µl	: Micro liter
µmol	: Micromole
et. al.	: et alliori (and others)
eg	: Exempli gratia (for example)
etc.	: et cetra (and the rest)
ELISA	: Enzyme Linked Immune Sorbent Assay
FAO	: Food and Agricultural Organization
g	: Gram
Gliclazide	: Type 2 Gliclazide treated group
Hb	: Haemoglobin
HDL	: High Density Lipoprotein
LDL	: Low Density Lipoprotein
OGTT	: Oral Glucose Tolerance Test
ie	: id est (That is)
ip	: Intraperitoneal
l	: Litre
mg	: miligram

ml	: mililitre
mmol/l	: Milimole per litre
ng	: nanogram
STZ	: Streptozotocin
WHO	: World Health Organization
ZAFME	: Zanthoxylum armatum fruits methanolic extract(ZAFME)
std	: Normal water control rat
NC	: diabetic water control rat
PC	: Gliclazide treated diabetic rat
TG1	: 25mg Zanthoxylum armatum fruits methanolic extract(ZAFME) treated rat
TG2	: 50mg Zanthoxylum armatum fruits methanolic extract(ZAFME) treated rat

CHAPTER ONE: INTRODUCTION

INTRODUCTION

1. Introduction

Diabetes is a complex, chronic illness requiring continuous medical care with multifactorial risk-reduction strategies beyond glycemic control (ADA, 2019). It occurs when the pancreas does not produce enough insulin (a hormone that regulates blood sugar, or glucose), or when the body cannot effectively use the insulin it produces (WHO, 2016). Diabetes describes a group of metabolic disorders characterized and identified by the presence of hyperglycaemia in the absence of treatment. The heterogeneous aetio-pathology includes defects in insulin secretion, insulin action, or both, and disturbances of carbohydrate, fat and protein metabolism. The long-term specific effects of diabetes include retinopathy and neuropathy, among other complications. People with diabetes are also at increased risk of other diseases including heart, peripheral arterial and cerebrovascular disease, obesity, cataracts, erectile dysfunction, and nonalcoholic fatty liver syndrome. They are also at increased risk of some infectious diseases, such as tuberculosis. Diabetes may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. Genital yeast infections frequently occur. The most severe clinical manifestations are ketoacidosis or a non-ketotic hyperosmolar state that may lead to dehydration, coma and, in the absence of effective treatment, death (WHO, 2019).

Type 2 diabetes is occurred due to a progressive loss of β-cell insulin secretion frequently on the background of insulin resistance (ADA, 2019). Type 2 diabetes accounts for the vast majority of people with diabetes around the world. Symptoms may be similar to those of type1 diabetes, but are often less marked or absent. As a result, the disease may go undiagnosed for several years, until complications have already arisen. For many years, type 2 diabetes was seen only in adults but it has begun to occur in children (WHO, 2016).

Natural products and their derivatives have been successful source of bioactive molecules in medicines much before the advancement of other modern therapeutics in the post-genomic era (Jadhav et al., 2012). Studies conducted in several developed countries reported that almost half to two thirds of the population affected with diabetes use complementary and alternative medicine to control the condition (Ceylan et al., 2009). The World Health Organization has

recommended and encouraged the use of alternative therapy especially in countries where access to the conventional treatment of diabetes is not adequate (WHO 1996). Medicinal herbs are expected to have a similar degree of efficacy without the troublesome side effects associated with conventional drug treatment (Shruthi et al., 2012). A multitude of herbs and medicinal plants and some compounds purified from them have been studied for the treatment of diabetes throughout the world as they might provide a basis of new synthetic antidiabetic analogues with potent activity (Grover et al., 2002). Plants which have been shown to have hypoglycemic action, act on blood glucose through different mechanisms. Some of them may inhibit endogenous glucose production (Eddouks et al., 2004) or interfere with gastrointestinal glucose absorption (Musabayane et al., 2006) and some may have insulin-like substances (Collier et al., 1987). World ethnobotanical information regarding medicinal plants reports almost 800 plants being used in the control of diabetes mellitus (Alarcon-Aguilara et al., 1998). There is much need to explore such resources for the development of new medicines to control or treat diabetes.

The liver plays a vital role in physiological functions that include oxidation, reduction, hydrolysis, conjugations, sulfation and acetylation in detoxification along with carbohydrate, lipid and protein metabolism of the body. If it did not function normally to regulate body metabolism, which reflects in alterations in different enzymes, hormones and protein levels. This will affect the health conditions of the human or any living organism leading to mortality. Therefore, it is very important to maintain a healthy liver to have a healthy body with effectively functioning organs.

The complex and bi-directional relationship linking the liver and diabetes has recently gained intense new interest. Fatty liver and diabetes share insulin resistance as their chief pathogenic determinant. Closing the circle, special emphasis is given to biochemical pathways and clinical evidence supporting the role of type 2 diabetes as a risk factor for the development of progressive liver disease, including non-alcoholic steatohepatitis, cirrhosis and primary liver cancer. Non-alcoholic fatty liver disease as a risk factor for the development of type 2 diabetes which is, in turn, a major contributor to progressive liver disease (Paola et al., 2013).

INTRODUCTION

Nowadays, a variety of factors cause injury to different organs of the body including liver and these include microbial infections, environmental pollutants and toxic chemicals. In recent studies, hepatotoxic chemical or drugs are found to cause more liver diseases compared to spontaneous liver diseases. Simultaneously, oxidative stress also plays an important role in damaging liver tissue and it is one of the major causes for liver damage around the world. Many drugs used to treat different diseases produce therapeutic benefit but also simultaneously cause side effects that at times lead to different organ damage in the body. Antibacterial drugs on longterm usage become ineffective due to microorganisms acquiring resistant to these drugs. The antibioticresistant microorganisms are a huge threat to global human health. In this regard, identification of new drugs from various sources is very important to control the increasing disease burden including liver diseases and side effects of existing drugs. Identification of new active metabolites from medicinal plants has been proved to be the best approach since older days, mainly herbal medicine has been the basis for current day drugs. Indian Ayurveda, Chinese traditional medicine, Traditional Korean medicine, Unani medicine, Traditional African medicine were mainly based on medicinal plants. In recent studies, various medicinal plants were reported to possess biological activities, bioactive metabolites, precursors for new bioactive molecules and therapeutic potential against several diseases including hepatic injury. However, many more medicinal plants still need to be investigated for potential biological activities that these might possess (Talluri et al., 2018).

Zanthoxylum armatum, commonly called Timur in Nepal (English: Nepal pepper or prickly ash), is an important medicinal plant. Eight species of Zanthoxylum have been reported from Nepal till now: *Z. acanthopodium*, *Zanthoxylum armatum*, *Z. floribunda*, *Z. nepalense*, *Z. nitidum*, *Z. oxyphyllum*, *Z. simularis* and *Z. tomentellum* (DPR, 2011a, 2016; Rajbhandari et al., 2015). Even though eight species of Zanthoxylum, only five species have been accepted taxonomically according to The Plant List. *Z. nepalense* is classed as an unresolved name, while *Z. floribunda* and *Z. simularis* are not recorded there (The Plant List, 2013). Among these species reported from Nepal, *Zanthoxylum armatum* is the most common and one of the 30 medicinal plants of the country, which has been prioritized by the government of Nepal for economic development with a high emphasis on cultivation and agrotechnology development (DPR, 2006). The different parts of the plants viz. leaves, fruits, stem, bark, seeds have been used in several indigenous

medicinal practices as carminative, antipyretic, appetizer, stomachic, toothache, dyspepsia (Manandhar, 2002; Kala et al., 2005; Singh et al., 2016). A wide array of chemical compounds including alkaloids, flavonoids, lignins, coumarins, phenols, terpenoids have been found in this plant. These compounds are responsible for various biological activities like antioxidative, antimicrobial, antiviral, hepato-protective, insecticidal/larvicidal etc., which have been demonstrated by several pharmacological studies. There is a huge demand of *Zanthoxylum armatum* in both domestic and international market due to which the market price has been escalating in the last two decades (Hertog and Wiersum, 2000). Despite of the species' importance, a comprehensive review on *Zanthoxylum armatum* is still not available. Hence an effort has been made to gather all the fragmentary information of *Zanthoxylum armatum* regarding the uses, phytochemistry, pharmacology and to analyze the current state of knowledge and possible opportunities that can be tapped for the overall benefit of the rural communities. It is important to establish a strong linkage between the traditional knowledge and modern researches to authenticate the ages old traditional ethno medicinal practices. Various parts of this *Z. armatum* are used in the preparation of tooth powder and medicinal preparations. The studies on this plant during the last few decades show that these plants contain various useful pharmacological active compounds (Singh et al., 2011).

The evidences are available where *Z. armatum* leaves water extract was tested for anti-diabetic potential in animals. The methanol extract of *Z. armatum* (fruit, bark and leaves) at a dose of 500 mg/kg showed significant effect on the glucose tolerance of mice and the extracts also showed reduction in the fasting blood glucose levels of the norm glycemic mice (Alam et al., 2018). The experiments demonstrated that *Z. armatum* water extract possess anti-diabetic activity in *in-vivo* procedure using mice (Rynjah et al., 2018) . In another experiment, the hydromethanolic extract of bark of *Z. armatum* was evaluated for its antidiabetic activity in streptozocin induced diabetes in rats. The total cholesterol, triglycerides, low density lipoprotein, very low density lipoprotein were also monitored (Karki et al., 2014). Therefore, there are enough evidence available for testing *Z. armatum* for anti-diabetic potentials in animal models.

The *in vitro* antioxidant activity of the *Z. armatum* has been reported in various models. A number of alkaloids viz. berberine, dictamnine, magnoflorine, xanthoplanine, sikimmianine and

fagarine have been reported from its stem-bark, wood and roots. The stem bark and leaves also contain an essential oil and resins. Experimental evidences show that free radicals are reported to be involved in the pathogenesis of liver injury. It is established that, plants having antioxidant property also exert hepatoprotective action (Talluri et al., 2018).

Bangladesh is a developing country in which a significant number of people have type 2 diabetes and here a number of people live below poverty line (Afroz et al., 2019). They could not meet up their daily nutritional requirement and a substantial population remains malnourished. As *Zanthoxylum armatum* have potential therapeutic or prophylactic effects, therefore, development of numerous *Zanthoxylum armatum* fruits extracts are badly required to treat different diseases especially type 2 diabetes. These natural medicines would be able to confer health benefits and lowering blood glucose level as well as liver enzymes viz. SGPT & SGOT of type 2 diabetes patients in Bangladesh.

The purpose of present study was to investigate the effect of *Zanthoxylum armatum* fruits methanolic extract (ZAFME) during the progression of streptozotocin induced type 2 diabetes in rats in comparison with standard drug, gliclazide. Literature reveals no such research work has been conducted so far. Therefore, the present study was undertaken with the following objectives:

1. To study the effect of *Zanthoxylum armatum* fruits methanolic extract on blood glucose, lipid profile, liver glycogen in neonatal streptozotocin (STZ) induced type-2 diabetic rats.
2. To determine the levels of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) in neonatal streptozotocin (STZ) induced type-2 diabetic rats.

CHAPTER TWO: LITERATURE REVIEW

LITERATURE REVIEW

2. Literature Review

2.1 General description on *Zanthoxylum armatum*

Zanthoxylum armatum is a small aromatic tree or large shrub up to 6m high. Branches are glabrous, usually armed with straight or slightly compressed, reddish brown stipular spines. The leaves are imparipinnate with 3–5 pairs of leaflets, elliptic-lanceolate, acuminate, base rounded or cuneate, sessile, margins usually entire, with a large gland associated with each tooth. The petiole and rachis are often winged between leaflets and sometimes bearing a spine at the point of insertion. Inflorescences are terminal panicles on short lateral shoots. Flowers are minute and polygamous, borne on short cymes. Male flower has 6–8 stamens, filaments 2mm arranged around globose pistillode. Female flower has 1–3 ovoid-subglobose carpels with two ovules attached to inner angle of axis. Fruit is a small drupe, reddish, ovoid and glandular warted, splitting into two when ripe (Fig. 1). Fruits contain single rounded and shining black seeds, 2–3mm in size (Grierson and Long, 1991; Nair and Nayar, 1997) Barkatullah et al. (2014) studied the anatomical characters of the leaf, stem bark and fruit of *Zanthoxylum armatum*. The internal structure of leaf shows a single layer of epidermis and palisade mesophyll. The vein-islets are squarish, elongated, polygonal or irregular with forked and unforked vascular branches. The internal structure of leaf was unique with the complete absence of any kind of trichomes or any other appendages. Nine types of stomata were recorded, among them brachparatetracytic was the most frequent one. Special leaf epidermal feature, the stomatal cluster was also observed. Bark and fruit anatomy of *Z. armatum* showed different tissue arrangement. The seed was non endospermic and contains an elongated embryo. Oggero et al. (2016) also studied the anatomical characters of the leaf and stem of *Zanthoxylum armatum* from central Argentina. The peculiar characteristics are the presence of secretory cavities, glandular trichomes and hypostomatic leaves.

Zanthoxylum armatum is found in hot valleys of subtropical to temperate Himalayas (Kashmir to Bhutan), north-east India and Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, Bhutan, Japan, North & South Korea, north Vietnam, Taiwan, Lesser Sunda Islands, Philippines, Malaya peninsula and Sumatra (Nair and Nayar, 1997). In Nepal, it is distributed from west to east at an

elevation range of 1000–2500m in open places or in forest undergrowth (DPR, 2007). The distribution range of *Zanthoxylum armatum* in Nepal based on herbarium specimens deposited at National Herbarium (KATH) and Tribhuvan University Central Herbarium (TUCH). The plant grows well in open pastures, wastelands and secondary scrub forests with adequate rainfall. Moist areas with deep soils exposed to sun and degraded slopes, shrub lands, natural forests and wastelands are the suitable habitat for *Zanthoxylum armatum*. For the cultivation of this species, clay or loam soil with high organic matter is preferable. The flowering starts on five year old plants in April-May and fruiting in August-October and can be harvested from October to January. Flowering is greatly affected by hails and storm (Kunwar and Pokharel, 2012).

Fig 2.1. Flower of *Zanthoxylum armatum*

It is generally propagated through seeds, but also from vegetative parts through soft wood cuttings. Natural regeneration usually occurs through seeds but the seeds undergo strong dormancy and may take few months to years for germination. Freshly harvested seeds are best for the large-scale cultivation. The seeds are sown in August-September in polybags in nursery or main field. The seeds germinate in 20–30 days after sowing. Stem cuttings may also be planted in the nursery during monsoon in July-August. The plants are ready for harvest after three years of plantation and the average annual yield of a five years plant is about 3.5 kg (ANSAB, 2011). The crop is generally free from any disease, insect or nematode attack, and physiological disorders. However Tara et al. (2011) investigated 7 insect pests on *Zanthoxylum armatum* that mostly caused the defoliation.

Parts used- Stem bark, fruits, and seeds

LITERATURE REVIEW

Synonyms:

Bengali :Gaira

Burmese : Gawra Kha Nan Nan, Teza Bo

Chinese : Ci Zhu Ye Hua Jiao, Qin Jiao (Taiwan), Zhu Ye Jiao

English : Bamboo-Leaved Prickly Ash, Nepal Pepper, Prickly Ash, Prickly Ash bark, Toothache Tree, Winged Prickly Ash, Winged Prickly Ash, Prickly Ash

German : Nepal pfeffer

Japanese : Fuyu Zanshou, Fuyu-Sansh

Korean : Gae San Cho

Thai : Mak Kak

Hindi : Tejphal, Tumru, Darmar, Trimal, Nepali dhaniya

Manipuri : Mukthrubi

Kannada : Dhiva, Tumburudu, Jimmi

Malayalam : Tumpunal, Tumpuni

Marathi : Chirphal, Naepaali dhane

Mizoram : Arhrikreh

Oriya : Arhrikreh, Ranabelli

Pashtu : Dambara

Sanskrit : Saurabha, Tejovati, Tumbaru, Vanaja

Tamil : Tumpunalu

Telugu : Gandhalu, Konda-kasimi, Konda, Kaasimanda

Urdu : Dambrary, Tamur

2.2 Taxonomy of *Zanthoxylum armatum*

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Viridaeplantae

Phylum: Tracheophyta

Subphylum: Euphyllophytina

Infraphylum: Radiatopses

Class: Magnoliopsida

Subclass: Rosidae

Superorder: Rutanae

Order: Rutales

Suborder: Rutineae

Family: Rutaceae

Genus: Zanthoxylum

Species: *Zanthoxylum armatum*

2.3. Phytochemistry

Various phytochemical constituents like terpenoids , flavonoids, alkaloids, phenolics, lignins, coumarins, glycosides and benzoids, steroids, fatty acids, alkenoic acids, amino acids have been extracted from different parts of the plant i.e. seed, leaf, fruit, root and bark (Li et al., 2006; Tiwary et al., 2007; Negi et al., 2011, 2012; Waheed et al., 2011; Bachwani et al., 2012; Joshi and Gyawali, 2012; Barkatullah et al., 2013; Brijwal et al., 2013 Bharti and Bhushan, 2015; Kayat et al., 2016; Singh et al., 2016). Monoterpenes like linalool and limonene are the major constituents of the essential oil. Seeds contain hydroxylic (4Z) enolic acid and various volatile compounds (Ahmad et al., 1993). The various alkaloids, flavonoids, flavonol glycosides, lignins, phenolics, sterols, terpenoids, fatty acids, alkenic acids, amino acids, various aromatic and volatile and number of other compounds have been identified and isolated from *Zanthoxylum armatum* essential oil in good quantity (Waheed et al., 2011; Bhatt et al., 2018). Gas chromatography-mass spectrometry (GC-MS) analysis reveals that the hexane extract of fruits contains 36 different chemical compounds (Kayat et al., 2016). The essential oil extracted through hydro-distillation through GC-MS revealed the major constituents as beta-Linalool (53.05%), Bergamot mint oil (12.73%), alpha-Limonene diepoxide (11.39%), alpha- pinene (4.08%), beta- Myrcene (3.69%) and D-Limonene (3.10%) (Barkatullah et al., 2013). A number of alkaloids and coumarins have been isolated and reported from the various parts of *Zanthoxylum armatum*: berberine (stembark), alkaloids (g-fagarine, b-fagarine, magnoflorine, laurifoline, nitidine, chelerythrine, tambetarine and candicine), coumarins (xanthyletin, zanthoxyletin, alloxanthyletin), and resin, tannin and volatile oil (Bachwani et al., 2012; Joshi and Gyawali, 2012). A new amide designated as armatamide along with two lignans, asarinin

and fargesin, alpha and beta-amyrins, lupeol, and beta-sitosterol-beta-D-glucoside have been isolated from the bark of *Zanthoxylum armatum* (Kalia et al., 1999). From the alcoholic extract of the stem bark, a new flavonoidal glycoside has also been isolated (Sati et al., 2011a). The main components of the oil are oleic acid, palmitic acid, linoleic acid methyl ester, limonene and linalool (Shah, 1991; Negi et al., 2012; Kayat et al., 2016). The essential oil from the seeds consists entirely over 85% of the hydrocarbon 1-α-phellandrene and also a small quantity of linalool and an unidentified sesquiterpene (Waheed et al., 2011). The stems and roots contain α-amyrin, α-sitosterol, L-asarinin, L-planinin, and zanthobungeanine (Verma and Khosa, 2012). Bark yields a bitter crystalline principle, identical to berberine, dictamnine, volatile oil and resin. The carpels yield a volatile oil, resin, yellow acid principle, and crystalline solid body, xanthoxylin (CSIR, 2005). Table 2 shows the different secondary compounds present in *Zanthoxylum armatum*.

2.4 Ethnobotanical uses / importance

Zanthoxylum armatum has been used extensively in traditional indigenous medicinal practices in Nepal by different ethnic communities. Several ethnobotanical studies have documented the various ethnomedicinal uses in different types of ailments. The seeds and barks of *Z. armatum* are used as aromatic, carminative, tonic in fever, dyspepsia (Anonymous, 1970). In stomach problems, the seeds powder is taken with warm water. The fruits and seeds are used for curing cholera, tooth ache and as leech repellant (Shrestha, 1985, 1988; Manandhar, 1987; Joshi and Edington, 1990; Joshi and Joshi, 2000; Balami, 2004). The fruits are used in fever, cold, cough, indigestion and as tonic (DPR, 1983). The bark, thorns and fruits are used in fish poisoning (Kunwar et al., 2009, 2013; Joshi, 2004; Malla et al., 2014). The seeds are chewed to cure toothache, added in vegetables for detoxification. The liquid (steam) collected after the fermentation of seeds are taken twice a day to cure tuberculosis. The seeds cooked with water, wheat flour and oil is taken during edema (Subedi, 2017). 5–6 powdered seeds are taken orally with lukewarm twice daily for one week in malfunction of the liver (Rai and Pokharel, 2006). The dried seeds can act as effective pesticide against small insects of wheat plants. The paste of its seeds and leaves of Artemisia vulgaris are applied to wooden house and furniture to repel termites and other wood eating insects (Turin, 2003). It is an important plant having various medicinal, pharmaceutical, biological properties. Fruits are used for toothache, dyspepsia, as a

carminative and for stomachache. Fruits are used as condiment and flavoring agent (Anonymous, 1970; Arshad and Ahmad, 2004; Iqbal and Hamayun, 2005; Abbasi et al., 2013). The branches, barks, fruits and seeds are extensively used as a carminative, stomachic and anthelmintic, branches are used as toothbrush. In Nepal, the fruit decoctions and berries are used for abdominal pain, carminative, antispasmodic, rheumatism, skin diseases, cholera, diabetes and asthma (Singh et al., 2016). Fresh fruits are used as spices and also for making pickles (Joshi, 2000; Manandhar, 2002; Balami, 2004; Malla et al., 2014) The fruits and seeds are employed as an aromatic tonic in fever, dyspepsia, tooth ache, stomach ache, and expelling roundworms (Verma and Khosa, 2012; Tiwary et al., 2007; Kalia et al., 1999; Rajbhandari et al., 2001; Uprety et al., 2010). Depending upon the environmental condition, the fruits may contain essential oil that ranged from 2% to 7% with an average yield of 5% (Manandhar, 2002; Paudel et al., 2017). The volatile oil is employed as an antidiarrheal, antiseptic, deodorant and anticatarrhal (Bhattacharya and Zaman, 2009; Bhattacharya et al., 2010; Neetu et al., 2001). The pharmaceutical companies generally use the fruits for making different types of toothpaste and gel. The different ethnomedicinal uses of *Zanthoxylum armatum* is presented in Table 1. The essential oil, extracted from the fruits is commercially known as Zanthoxylum oil, which is highly valued for its vibrant aroma and used in perfumeries and has a high demand in international markets (DoF, 2014). It is frequently used in different aroma therapy because of its soothing effect. Almost all parts of the plant are aromatic and hence, supposed to possess essential oil. The essential oil composition has much more importance regarding the medicinal properties and its constituents (Bhattacharya and Zaman, 2009).

2.5 Pharmacological study:

2.5.1 Antidiabetic activities

The antidiabetic potential of *Zanthoxylum armatum* have been demonstrated by experiments by different authors There are reports of antidiabetic studies on Zanthoxylum plants for example *Zanthoxylum zanthoxyloides* leaves exhibits antidiabetic and hypolipidemic effects (Slei et al.,2012). Similarly, Z. armatum bark showed antidiabetic activity on streptozotocin induced diabetes in rats (Karki et al.,2014). The methanol extract of *Z. armatum* (fruit, bark and leaves) at a dose of 500 mg/kg showed significant effect on the glucose tolerance of mice and the extracts also showed reduction in the fasting blood glucose levels of the norm glycemic mice, thus

revealing the hypoglycemic nature of the extracts. The effect was more pronounced for the methanol extract of *Z. armatum* leaves (Alam et al., 2018).

2.5.2. Hepatoprotective activities

The ethanol extract of leaves *Zanthoxylum armatum* was used to study the in-vivo hepatoprotective effect against Carbon tetrachloride (CCl4) induced liver damage in Wister albino rats. Silymarin (100mg/kg of body weight) was used as a positive control. CCl4 intoxication in normal rats elevated the levels of SGOT, SGPT, ALKP, SBLN and liver inflammation were observed significantly indicating acute hepatocellular damage and biliary obstruction. Oral administration of the extract at 500mg/kg showed a significant ($P < 0.001$) decrease in all the SGOT, SGPT, ALKP, SBLN levels and liver inflammation, by normalizing the elevated levels of the hepatic enzymes. The results obtained support the use of this plant for the treatment of hepatitis in oriental traditional medicine (Verma and Khosa, 2012). In another experiment, the hepatoprotective activity of ethanolic extract of bark of *Zanthoxylum armatum* in CCl4 induced hepatotoxicity in male Wister rats was examined. 1:1 of CCl4 in olive oil administration caused a significant increase in the serum activities of ALT, AST, ALP, DBil and TBil. The oral administration of doses (ethanol extract) at 100, 200, and 400mg/kg once daily for 7 days significantly reduced the above elevated parameters. However, treatment with 400 mg/kg showed significant hepatoprotective activity that was comparable to silymarin (25mg/kg) (Ranawat et al., 2010).

2.5.3. Antibacterial activities

In-vitro assessment of antibacterial activity of the ethanolic and nhexane extracts of leaves, fruits and bark of *Zanthoxylum armatum* was evaluated against different bacterial strains: *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Streptococcus viridians* through agar well diffusion method (Barkatullah et al., 2012). The wells were supplied with 100 µl of 3 mg/mL of respective extract dissolved in dimethyl sulfoxide (DMSO). DMSO and Ciprofloxacin (10 µg well−1) were used as negative and positive controls respectively. The maximum inhibition was exhibited by the ethanolic extract of the fruits extract against *M. luteus* with (21.33 ± 0.41mm ZOI) and *P. multocida* (18.33 ± 0.41 mm), followed by the ethanolic extract of the leaves against M. luteus (18.00 ± 0.71 mm), P. multocida (18.00 ± 0.71 mm) and finally by hexane extract of the fruits

against *M. luteus* (19.67 ± 0.41 mm). The ethanolic extract of the bark showed moderate activity against all tested bacteria while the hexane extract of the bark extract was found active against *M. luteus* (20.33 ± 0.41 mm), *P. multocida* (17.67 ± 0.41 mm). The minimum inhibitory concentration (MIC) values for most of the bacterial species were found to be 0.65 µg/ mL. The most resistant bacterial strain was *B. subtilis* with a maximum MIC value ranging from 1.25 to 5 mg/mL while the lowest MIC was observed for *S. aureus* ranging from 0.65 to 2.5 mg/mL. In another experiment, in-vitro antibacterial activities of the leaf essential oil and methanol extract of *Zanthoxylum armatum* were tested against different bacteria using agar well diffusion method. The oil (diluted with DMSO 1:1 and 20 µl well−1) exhibited strong activity against *Micrococcus luteus, Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* with zones of inhibition ranging from 15 to 21 mm; the maximum was 21mm against *Micrococcus luteus* while Pseudomonas aeruginosa showed moderate activity with 7mm zone of inhibition. *Pseudomonas aeruginosa* was least sensitive to the oil with MIC value> 1000 µg/mL while it was 62.5 µg/mL for *Micrococcus luteus* and 125.5 µg/mL for *Staphylococcus aureus. Escherichia coli* and *Bacillus subtilis* were also sensitive to the oil with MIC values of 250 µg/mL and 500 µg/mL respectively. Methanol extract (2 mg well−1)) was found to be ineffective against all the tested bacterial strains. Chloramphenicol (10 µl well−1) was used as control (Guleria et al., 2013). The antibacterial properties may be attributed the collegial effects of several compounds present in their crude extracts. These experiments substantiates further research for exploiting the possible uses of *Zanthoxylum armatum* for treating different bacterial diseases like urinary tract infection and skin infection, diarrhea and dysentery.

2.5.4. Antifungal activities

In-vitro anti-mycotic test of the essential oil obtained from the leaves of *Zanthoxylum armatum* exhibited remarkable activities against various fungal strains like *Trichophyton longifilis, Candida albicans, Fusarium solani, Microsporum canis, Aspergillus flavus* and *Candida glabrata.* Maximum effect as a percent inhibition of mycelial growth was observed against *Candida albicans* (66.67 ± 0.57%) followed by *Aspergillus flavus* (55.33 ± 0.57%) and *Fusarium solani* (46.33 ± 0.33%) at 125 µg/mL concentration. DMSO and Miconazole were used as negative and positive control respectively (Barkatullahet al., 2013). In another experiment, the essential oil from the leaves showed antifungal activities against three crop pathogens namely *Alternaria alternata, Alternaria brassicae, Curvularia lunata* with antifungal

indices of 35.6 ± 1.49%, 14.5 ± 0.36% and 42.0 ± 1.63% respectively. Similarly antifungal indices of methanol extract of leaves were 47.4% and 51.4% against *Alternaria alternata* and *Curvularia lunata* respectively. The methanolic extract did not show any activity against *Alternaria brassicae* at 2 mg/mL concentration (Guleria et al., 2013). The antimycotic potential of crude methanol extract could be because of the phenolic and flavonoid compounds present in the leaves. Several in-vitro antifungal studies verified the antimycotic potential of *Zanthoxylum armatum* against various fungal strains; *Aspergillus parasiticus* (Dube et al., 1990), *Candida albicans, Cryptococcus neoformans* (Goel et al., 2002), *Microsporum gypseum, Trichophyton mentagrophytes* (Dikshit et al., 1986), *Sclerotium rolfsii, Rhizoctonia bataticola* (Sharma et al., 2009), Alternaria brassicicola (Parajuli et al., 2005), Bipolaris sorokiniana (Manandhar and Tiwari, 2005). These experiments necessities further specific researches for the possible use of *Z. armatum* against various crop pathogens.

2.5.5. Antioxidant activity

The antioxidant potential of *Zanthoxylum armatum* have been demonstrated by several in-vitro/in-silico antioxidant experiments by different authors (Batool et al., 2010; Upadhyaya and Kumar, 2010; Negi et al., 2012; Prakash et al., 2012; Barkatullah et al., 2013; Guleria et al., 2013; Karmakar et al., 2015; Kanwal et al., 2015 etc.). However such studies are not considered in this review as per the skeptical judgment of such studies by scientific journals. All plants possess antioxidant activity so it is insignificant unless evidenced by in-vivo studies (Gafner, 2018). Karmakar et al., (2015) carried out in-vivo antioxidant assay of methanol extract of leaves of *Zanthoxylum armatum* in male Wister Albino rats. The test animals were orally administered with the extract at a dose of 100 and 200 mg/kg body weight, whereas the normal control animals were given distilled water and positive control group was given standard Vitamin E. After five days, the test animals were given a mixture of 1:1 CCl4 and olive oil for 2 days. The activities of SOD, CAT and GSH were decreased significantly ($P < 0.01$) in normal control groups, whereas lipid peroxidation level was found to increase in EAT bearing control group as compared to normal animals. Administration of the extract significantly enhanced the activities of antioxidant enzymes (SOD, CAT and GSH) in the treated animals. Treatment at the dose of 100 and 200 mg/kg increased the levels of SOD and CAT significantly ($P < 0.05$ and $P < 0.01$) and GSH was also increased, whereas the lipid peroxidation level was decreased at 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.01$) respectively. The excessive production of free radicals might

cause cell and tissue damage, resulting in aging and untimely cell death (Hsieh et al., 2009). The prominent antioxidant enzymes to inhibit free radical formation are SOD, CAT and GSH (Liu et al., 2013). The results obtained in the present study indicated the potent antioxidant activity of *Zanthoxylum armatum*. Further research could validate the use of the plant as a natural antioxidant.

2.5.6. Anti-inflammatory activity

In-vivo anti-inflammatory activity of ethanolic extract of stem bark of *Zanthoxylum armatum* was evaluated by carrageenan induced paw edema method in male Wister rats (Sati et al., 2011b). The degree of inhibition was 19.12%, at 250 mg/kg dose after 4 h of administration. Ibuprofen (10 mg/kg of body weight) was used as positive control. Fruits extract also showed inhibition of carrageenan that induced paw edema in Wister rats (Mehta et al., 2011). It has analgesic activity also due to the presence of lignan components (Guo et al., 2010).

2.5.7. Antispasmodic effect

The crude extract of *Zanthoxylum armatum* was used to study the *in vivo* spasmolytic effects against castor-oil-induced diarrhea in mice. Pre-treatment of animals with the extract showed 20% protection from diarrhea at 300 mg/kg and 60% protection at 1000 mg/kg. Loperamide (10 mg/kg) was used as positive control (Gilani et al., 2010). In another experiment, the essential oil of leaves of *Zanthoxylum armatum* was evaluated for possible antidiarrheal effect on spontaneous and potassium chloride induced contracted smooth muscle of the isolated rabbit jejunum. The spasmolytic effect of the oil started from 0.03 mg/ mL and showed 100% effect at 10 mg/mL dose. The extracts relaxed the contracted muscle, suggesting the possible mode of action of this plant as either blocking the release of stored calcium from the sarcoplasmic reticulum or blocking the calcium channel (Barkatullah et al., 2013).

2.5.8. Inhibition of keratinocyte growth

The methanol extract of the bark of *Zanthoxylum armatum* was evaluated for the anti-proliferative activity against the growth of rapidly multiplying human keratinocytes (HaCaT cells). The HaCaT keratinocyte proliferation assay and determination of cell growth was carried out. The concentrations of the extracts used were between 0.008 and 0.4 mg/mL. The extract significantly inhibited the growth of keratinocytes. It was found to be highly active with an IC50 value of 11 mg/mL (Kumar, Müller, 1999)

2.5.9. Cytotoxic and phytotoxic effects

Barkatullah et al. (2013) carried out in-vitro toxicity bioassay in Brine shrimp to investigate the preliminary cytotoxic potential of the crude ethanolic and n-hexane extract of leaf, bark, fruits and leaf essential oil of *Zanthoxylum armatum*. The concentrations tested were 10, 100 and 1000 µg/ml. The oil exhibited remarkable mortality rate (100%) at a dose of 1000 µg/ml with LC50 value 15.90. However, a dose of 1000 µg/ml clearly is of no therapeutic relevance and concerns remain about today's the relevance of this test system. Similarly in another experiment, the ethanolic extract and subsequent fractions of the fruits of *Zanthoxylum armatum* were evaluated for Brine shrimp lethality test using concentrations 5, 50, and 500 mg/mL. Etoposide was used as standard cytotoxic drug. The crude extract exhibited significant toxicity with LC50 value of 6.66 ± 1.1 mg/mL, which probably indicates the distribution of synergistic activity of compounds within these two fractions. Similarly, the LC50 values of chloroform and aqueous-methanol fraction was 21.4 ± 3.3 and 29.6 ± 3.9 mg/mL respectively. The n-hexane fraction, exhibited lower toxicity level with LC50 value greater than1000 mg/mL. (Alam and us Saqib, 2017). Further research could possibly find out the active biochemical compounds. In another experiment, different concentrations (1000, 100 and 10 mg/mL) of the crude extract, n-hexane, chloroform and aqueousmethanolic fractions of fruits of *Zanthoxylum armatum* was evaluated for in-vitro phytotoxic activity against Lemna minor L. Paraquat (0.9025 mg/mL) was used as positive control. Remarkable phytotoxicity was observed at the highest concentration (1000 mg/mL) causing complete inhibition of the plant growth. At 100 mg/mL concentration, the n-hexane extract caused complete inhibition while the crude extract and chloroform extract exhibited 63.3 ± 5% and 54.54 ± 7% inhibitions, respectively. Lowest effect was observed for the aqueous methanol extract with only 22.72 ± 4% inhibition (Alam and us Saqib, 2017).

2.5.10. Antiviral/antiprotozoal activity

Methanolic extracts of fruits of *Zanthoxylum armatum* (concentrations of 100, 50, 25, 12.5 and 6.25 mg/mL) have been investigated for in vitro antiviral activity against Herpes simplex virus type 1(HSV-1) and influenza virus A (100, 50, 25, 12.5 mg/mL) by dye uptake assay in the systems HSV-1/Vero cells and influenza virus A/MDCK cells. The extracts showed inhibition of HSV-1/ Vero cells, Influenza A/MDCK cells with CC50 value> 100 mg/mL and 36 mg/mL (Rajbhandari et al., 2009). The aqueous extract of the leaves showed antiprotozoal effect on Giardia lamblia and Plasmodium berghei (Goel et al., 2002).

2.5.11. Soothing effect on skin

The lipophilic extract with alcohol gives remarkable soothing effect based on inhibition of sensory irritation from sun bathing, shaving depilation, insect bites and chemical treatment (Guglielmini and Cristoni, 2002).

2.5.12. Mosquito repellant

In combination, *Zanthoxylum armatum* seed oil, vanillin and fruit oil of *Zanthoxylum piperitum* have been able to enhance repellent activity against female Aedes aegypti and the effect was compared with N,N diethyl-3methylbenzamide (DEET) repellent (Kwon et al., 2011). The essential oil was found to significantly repel mosquitoes at 0.57 mg/cm2 concentrations. viz. 445 min in mustard oil and 404 min in coconut oil
base (Das et al., 1999).

2.5.13. Larvicidal activities

Tiwary et al. (2007) studied in-vitro larvicidal activities essential oil of the seeds of *Zanthoxylum armatum* against three species of mosquitoes: *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*. The test doses used for the study were 200, 150, 100, 50, 25 and 10 ppm. It was found that *Culex quinquefasciatus* was the most sensitive with LC50 and LC95 values of 49 and 146 ppm respectively followed by *Aedes aegypti, Anopheles stephensi* with LC50 values in the range of 54–58 ppm. Temephos, chemical larvicide used commonly for controlling mosquito larvae, was used as positive control at a range of 0.005–0.1 ppm concentration. Similar larvicidal effects were also studied by Peng et al. (2009) against *Culex pipiens, Culex quinquefasciatus* and Zhang et al. (2010) against *Aedes albopictus* and *Culex pipiens*.

2.5.14. Allelopathic effects

Different concentrations (i.e., 2%, 5%, and 10%) of aqueous extracts of leaf, bark, and fruit pulp were found to have significant allelopathic effect on some important winter field crops (*Triticum aestivum, Hordeum vulgare, Brassica campestris and Lens culinaris*). A study carried out in Garhwal Himalaya region of India showed significant effects of these bioassays on germination and growth of all the test crops. In an average, 83.6%, 52.6%, and 84.9% reduction in radicle growth was observed in *Triticum aestivum, Hordeum vulgare and Lens culinaris*, respectively (Singh et al., 2007).

2.6 Epidemiology and global burden of diabetes

Diabetes is found in every population in the world and in all regions, including rural parts of low- and middle-income countries. The number of people with diabetes is steadily rising, with WHO estimating there were 422 million adults with diabetes worldwide in 2014. The age-adjusted prevalence in adults rose from 4.7% in 1980 to 8.5% in 2014, with the greatest rise in low- and middle-income countries compared to high-income countries. In addition, the International Diabetes Federation (IDF) estimates that 1.1 million children and adolescents aged 14–19 years have T1DM. Without interventions to halt the increase in diabetes, there will be at least 629 million people living with diabetes by 2045. High blood glucose causes almost 4 million deaths each year, and the IDF estimates that the annual global health care spending on diabetes among adults was US$ 850 billion in 2017. The effects of diabetes extend beyond the individual to affect their families and whole societies. It has broad socio-economic consequences and threatens national productivity and economies, especially in low- and middle-income countries where diabetes is often accompanied by other diseases(WHO, 2019).

2.7 Aetio-pathology of diabetes

It is now generally agreed that the underlying characteristic common to all forms of diabetes is the dysfunction or destruction of pancreatic β-cells. Many mechanisms can lead to a decline in function or the complete destruction of β-cells (these cells are not replaced, as the human pancreas seems incapable of renewing β-cells after the age of 30 years). These mechanisms include genetic predisposition and abnormalities, epigenetic processes, insulin resistance, auto-immunity, concurrent illnesses, inflammation, and environmental factors. Differentiating β-cell dysfunction and decreased β-cell mass could have important implications for therapeutic approaches to maintaining or improving glucose tolerance. Understanding β-cell status can help define subtypes of diabetes, and guide treatment(WHO, 2019).

2.8 Diabetes mellitus

Diabetes mellitus, more simply called diabetes, is a chronic condition that occurs when there are raised levels of glucose in the blood because the body cannot produce any or enough of the hormone insulin or use insulin effectively.1 Insulin is an essential hormone produced in the pancreas gland of the body, and it transports glucose from the bloodstream into the body's cells where the glucose is converted into energy. The lack of insulin or the inability of the cells to

respond to insulin leads to high levels of blood glucose, or hyperglycaemia, which is the hallmark of diabetes. Hyperglycaemia, if left unchecked over the long term, can cause damage to various body organs, leading to the development of disabling and life-threatening health complications such as cardiovascular disease, neuropathy, nephropathy and eye disease, leading to retinopathy and blindness. On the other hand, if appropriate management of diabetes is achieved, these serious complications can be delayed or prevented. The classification and diagnosis of diabetes are complex and have been the subject of much consultation, debate and revision stretching over many decades, but it is now widely accepted that there are three main types of diabetes, type 1 diabetes, type 2 diabetes and gestational diabetes (GDM). There are also some less common types of diabetes which include monogenic diabetes and secondary diabetes. Monogenic diabetes is the result of a single genetic mutation in an autosomal dominant gene rather than the contributions of multiple genes and environmental factors as seen in type 1 and type 2 diabetes. Examples of monogenic diabetes include conditions like neonatal diabetes mellitus and maturity-onset diabetes of the young (MODY). Around 1-5% of all diabetes cases are due to monogenic diabetes. Secondary diabetes arises as a complication of other diseases such as hormone disturbances (e.g., Cushing's disease or acromegaly), diseases of the pancreas (e.g., pancreatitis) or as a result of drugs (e.g., corticosteroids)(IDF atlas, 2017).

2.9 Complications of Diabetes

When diabetes is not well managed, complications develop that threaten health and endanger life. Acute complications are a significant contributor to mortality, costs and poor quality of life. Abnormally high blood glucose can have a life-threatening impact if it triggers conditions such as diabetic ketoacidosis (DKA) in types 1 and 2, and hyperosmolar coma in type 2. Abnormally low blood glucose can occur in all types of diabetes and may result in seizures or loss of consciousness. It may happen after skipping a meal or exercising more than usual, or if the dosage of anti-diabetic medication is too high.

Over time diabetes can damage the heart, blood vessels, eyes, kidneys and nerves, and increase the risk of heart disease and stroke. Such damage can result in reduced blood flow, which – combined with nerve damage (neuropathy) in the feet – increases the chance of foot ulcers, infection and the eventual need for limb amputation. Diabetic retinopathy is an important cause

of blindness and occurs as a result of long-term accumulated damage to the small blood vessels in the retina. Diabetes is among the leading causes of kidney failure.

Uncontrolled diabetes in pregnancy can have a devastating effect on both mother and child, substantially increasing the risk of fetal loss, congenital malformations, stillbirth, perinatal death, obstetric complications, and maternal morbidity and mortality. Gestational diabetes increases the risk of some adverse outcomes for mother and offspring during pregnancy, childbirth and immediately after delivery (pre-eclampsia and eclampsia in the mother; large for gestational age and shoulder dystocia in the offspring). However, it is not known what proportion of obstructed births or maternal and perinatal deaths can be attributed to hyperglycaemia. The combination of increasing prevalence of diabetes and increasing lifespans in many populations with diabetes may be leading to a changing spectrum of the types of morbidity that accompany diabetes. In addition to the traditional complications described above, diabetes has been associated with increased rates of specific cancers, and increased rates of physical and cognitive disability. This diversification of complications and increased years of life spent with diabetes indicates a need to better monitor the quality of life of people with diabetes and assess the impact of interventions on quality of life(WHO, 2016).

2.10 Classification of Diabetes

Diabetes can be classified into the following general categories (ADA, 2019):

1. Type 1 diabetes (due to auto immune b-cell destruction, usually leading to absolute insulin deficiency)
2. Type 2 diabetes (due to a progressive loss of b-cell insulin secretion frequently on the background of insulin resistance)
3. Gestational diabetes mellitus (GDM) (diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation)
4. Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation)

2.11 Type 2 diabetes

T2DM accounts for between 90% and 95% of diabetes, with highest proportions in low- and middleincome countries. It is a common and serious global health problem that has evolved in association with rapid cultural, economic and social changes, ageing populations, increasing and unplanned urbanization, dietary changes such as increased consumption of highly processed foods and sugarsweetened beverages, obesity, reduced physical activity, unhealthy lifestyle and behavioural patterns, fetal malnutrition, and increasing fetal exposure to hyperglycaemia during pregnancy. T2DM is most common in adults, but an increasing number of children and adolescents are also affected. β-cell dysfunction is required to develop T2DM. Many with T2DM have relative insulin deficiency and early in the disease absolute insulin levels increase with resistance to the action of insulin. Most people with T2DM are overweight or obese, which either causes or aggravates insulin resistance. Many of those who are not obese by BMI criteria have a higher proportion of body fat distributed predominantly in the abdominal region, indicating visceral adiposity compared to people without diabetes. However, in some populations, such as Asians, β-cell dysfunction appears to be a more notable prominent than in populations of European descent. This is also observed in thinner people from low- and middle-income countries such as India, and among people of Indian descent living in high-income countries. For most people with T2DM, insulin treatment is not required for survival but may be required to lower blood glucose to avert chronic complications. T2DM often remains undiagnosed for many years because the hyperglycaemia is not severe enough to provoke noticeable symptoms of diabetes. Nevertheless, these people are at increased risk of developing macrovascular and microvascular complications. Complications are a particular problem in young-onset T2DM – increasingly recognized as a severe phenotype of diabetes and associated with greater mortality rates, more complications, and unfavorable cardiovascular disease risk factors when compared to T1DM of similar duration. In addition, the response to oral blood glucose medications is often poor among young people with diabetes.

Many factors increase the risk of developing T2DM including age, obesity, unhealthy lifestyles and prior gestational diabetes (GDM). The frequency of T2DM also varies between different racial and ethnic subgroups, especially in young and middle-aged people. There are particular populations that have a higher occurrence of type 2 diabetes, for example Native Americans, Pacific Islanders, and populations in the Middle East and South Asia . It is also often associated

with strong familial, likely genetic or epigenetic predisposition . However, the genetics of T2DM are complex and not clearly defined, though studies suggest that some common genetic variants of T2DM occur among many ethnic groups and populations. Ketoacidosis is infrequent in T2DM but when seen it usually arises in association with the stress of another illness such as infection. Hyperosmolar coma may occur, particularly in elderly people. The specific aetiologies of T2DM are still unclear, and likely reflect several different mechanisms. It is likely that in future, subtypes will be created that may be classified under "other types" (see "Other specific types of diabetes")(WHO, 2019).

2.12 Managing Type 2 Diabetes

The starting point for living well with diabetes is an early diagnosis – the longer a person lives with undiagnosed and untreated diabetes, the worse their health outcomes are likely to be. Easy access to basic diagnostics, such as blood glucose testing, should therefore be available in primary health-care settings. Established systems for referral and back-referral are needed, as patients will need periodic specialist assessment or treatment for complications.

For those who are diagnosed with diabetes, a series of cost-effective interventions can improve their outcomes, regardless of what type of diabetes they may have. These interventions include blood glucose control, through a combination of diet, physical activity and, if necessary, medication; control of blood pressure and lipids to reduce cardiovascular risk and other complications; and regular screening for damage to the eyes, kidneys and feet, to facilitate early treatment. Diabetes management can be strengthened through the use of standards and protocols. Efforts to improve capacity for diagnosis and treatment of diabetes should occur in the context of integrated noncommunicable disease (NCD) management to yield better outcomes. At a minimum, diabetes and cardiovascular disease management can be combined. Integrated management of diabetes and tuberculosis and/or HIV/AIDS can be considered where there is high prevalence of these diseases(WHO, 2016).

2.13 Preventing Type 2 Diabetes

Effective approaches are available to prevent type 2 diabetes and to prevent the complications and premature death that can result from all types of diabetes. These include policies and practices across whole populations and within specific settings (school, home, workplace) that

contribute to good health for everyone, regardless of whether they have diabetes, such as exercising regularly, eating healthily, avoiding smoking, and controlling blood pressure and lipids.

Taking a life-course perspective is essential for preventing type 2 diabetes, as it is for many health conditions. Early in life, when eating and physical activity habits are formed and when the long-term regulation of energy balance may be programmed, there is a critical window for intervention to mitigate the risk of obesity and type 2 diabetes later in life.

No single policy or intervention can ensure this happens. It calls for a whole-of-government and whole-of-society approach, in which all sectors systematically consider the health impact of policies in trade, agriculture, finance, transport, education and urban planning – recognizing that health is enhanced or obstructed as a result of policies in these and other areas(WHO, 2016).

2.14 Plant material and Diabetes

Plants have been a good source of drugs. There are more than 800 plants that possess potent anti-diabetic activity (Alarcon *et al.,* 1998) and have been used as a dietary adjuvant or as a drug which have a traditional history in India. This treatment is the safe, cost effective and with least of side effect than that of synthetic hypoglycemic agent. The plant originated products act through two mechanism either insulinomimetic or as secretagogues. There are various types of phytoconstituents like alkaloids, flavonoids and saponins glycosides present in the plants responsible for its anti-diabetic activity. Alkaloids acts by inhibiting α –glucosidase and decrease glucose transport through intestinal epithelium. Flavanoids suppresses glucose level, reduces plasma cholesterol and triglyceride significantly and increases hepatic glucokinase activity probably by enhancing insulin from β cells. (Deepika *et al., 2013*).

Most of the drugs from plant sources are secondary metabolites which have no role in plant metabolisms but are postulated to play a significant role in the plant defense mechanism. A wide array of plant derived active principles representing numerous chemical compounds has demonstrated activity consistent with their possible use in the treatment of NIDDM (Bailey and Day, 1989; Marles and Farnsworth, 1995). There are about 200 pure compounds from plant sources reported to show blood glucose lowering effect. The compounds may be alkaloids, carbohydrates, glycosides, flavonoids, steroids, terpenoids, peptides and amino acids, lipids, phenolics, glycopeptides and iridoids. (L. Warjeet Singh, 2011).

Many herbs, spices, other plant materials and certain edible fungi or mushrooms; which are the blessings of nature, had been used from antiquity for the treatment of diabetes and inflammation. But their uses have been declined in occidental societies following the introduction of insulin and the anti-hyperglycemic sulphonylureas and biguanides. Unfortunately, they have a number of side effects and none of them have been fully successful in maintaining life-long euglycemia and controlling long-term microvascular and macrovascular complications of diabetes. As a result, a growing need has emerged for new oral antidiabetic drugs as an alternative therapy. The effective oral hypoglycemic agents appear to find a place in the plant kingdom, as several plants are known for their antidiabetic properties. Traditional antidiabetic plants might provide a useful source of new oral hyperglycemic compounds for development as pharmaceutical entities or as simple dietary adjuncts to existing therapies (Bailey *et.al.*, 1989). A total of more than 400 species were reported to display hypoglycemic effects, but few of them have been investigated scientifically (Bailey, *et.al.*, 1989). So far, more than 90 plants (e.g, *Trigonella foenumgraecum, Allium cepa, Hemidesmus indicus, Syzigium cumini, Murraya koegini* etc) for hypoglycemic properties have been screened from Bangladesh (BIRDEM). Herbal treatments are becoming increasing by popular as the herbal preparations have no or least side effects (Rajasekaran *et al*, 2001) The World Health Organization (WHO) has recommended further investigation in this area. These plants may not only provide useful dietary adjuncts to existing therapies, but may also represent a source of new hypoglycemic compounds for characterization and subsequent development of pharmaceutical modalities (Swanston *et al.*, 1990).

2.15 Preventing Mechanism of Zanthoxylum armatum against Type 2 diabetes:

α-glucosidase is the key enzyme in the digestion of carbohydrates in the surface membranes of intestine. α- glucosidase inhibitors suppress the postprandial hyperglycemia by retarding the liberation of D-glucose of oligosaccharides and disaccharides from dietary complex carbohydrates and therefore delay the glucose absorption (Gao et al.,2008). Acarbose, one of such inhibitors are approved in management of type 2 diabetes and for the treatment of obesity (Balfour et al.,1993). It is necessary to search for more effective and safe α-glucosidase inhibitors from natural materials, in order to develop antidiabetic agents. The extracts of *Z. armatum* showed very significant activity against alphaglucosidase and all the extract inhibited the enzyme with low concentration comparable with the standard drug acarbose. In vivo studies,

the extracts of *Z. armatum* lower the glucose level of alloxan induced diabetes to significant level.

2.16 Liver

The liver is an organ only found in vertebrates which detoxifies various metabolites, synthesizes proteins and produces biochemicals necessary for digestion. In humans, it is located in the right upper quadrant of the abdomen, below the diaphragm. Its other roles in metabolism include the regulation of glycogen storage, decomposition of red blood cells and the production of hormones. The liver is an accessory digestive organ that produces bile, a fluid containing cholesterol and bile acids, and an alkaline compound which helps the breakdown of fat. Bile aids in digestion via the emulsification of lipids. The gallbladder, a small pouch that sits just under the liver, stores bile produced by the liver which is afterwards moved to the small intestine to complete digestion. The liver's highly specialized tissue consisting of mostly hepatocytes regulates a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions. Estimates regarding the organ's total number of functions vary, but textbooks generally cite it being around 500. It is not yet known how to compensate for the absence of liver function in the long term, although liver dialysis techniques can be used in the short term. Artificial livers are yet to be developed to promote long-term replacement in the absence of the liver. As of 2018, liver transplantation is the only option for complete liver failure(Wikipedia).

2.17 Serum glutamic pyruvic transaminase (SGPT)

Serum glutamic pyruvic transaminase (SGPT) also known as Alanine Aminotransferase is one of the enzymes within the aminotransferases group and are among the most sensitive liver enzymes. The highest concentrations of the SGPT enzyme can be in the liver, with decreasing concentrations found in the kidneys, heart, skeletal muscle, pancreas, spleen and lung tissue respectively. SGPT measurements are used in the diagnosis of hepatic damage and diseases including viral hepatitis and cirrhosis.The normal concentration levels of SGPT in the blood are low, however, when damaged, the liver releases more ALT into the blood causing the concentration levels to rise. When diagnosing for hepatic damage, the root cause of the damage can be established, such as disease, drug, or injuryThe SGPT test not only allows for the

diagnosis of liver disease but also allows for the diagnosis of the root cause of the disease. It is often tested in combination with the aspartate aminotransferase (AST) test as part of the hepatic panel with alanine aminotransferase levels being higher in most types of liver disease.

2.18 Serum glutamic oxaloacetic transaminase (SGOT)

Serum glutamic oxaloacetic transaminase (SGOT), formerly known as Aspartate Aminotransferase (AST) is an enzyme found throughout the body. The high concentration levels of SGOT are found in the liver and lower concentration levels are found in heart cells, red blood cells, muscle tissues and other organs including the kidneys and pancreas. Elevated concentrations levels of SGOT in the blood are directly correlated to the severity of the tissue or organ damage and can signal myocardial infarction, hepatic disease, muscular dystrophy and organ damage. Excessive levels (10 times above the normal range) is indicative of liver damage including acetaminophen overdose, acute viral hepatitis or acute fulminant hepatitis, and tumor necrosis. Moderately high concentration levels of SGOT are indicative of alcohol abuse, hemolysis, heart damage including heart attack or heart failure, muscle injury including trauma or muscle dystrophy. Slightly elevated concentration levels of SGOT are indicative of cirrhosis, fatty change in the liver or mononucleosis. SGOT often tested in combination with the serum glutamic pyruvic transaminase (SGPT) test as part of the hepatic panel as ALT concentration levels are higher in most types of liver disease.

2.19 Hepatoprotective Mechanism of Zanthoxylum armatum

Paracetamol is a widely used medication for fever and pain because of its low cost and effectiveness, but its overdose and long-term usage cause adverse effects that include liver damage. Recently it was observed that drug-induced liver toxicity is one of the main causes of mortality and the mechanism of drug-induced liver damage was unclear. Some recent reports indicated that the liver damage is more due to free radical formation induced by excessive use of hepatotoxic drugs. These drugs affect organs functionality, enhance their metabolism leading to free radical formation and breakdown of macro molecules. Enhanced production of free radicals,

which are more unstable molecules, cause break down of cell membranes of cells including liver cells and alter molecular stabilization. In previous reports, Z. armatum extracts demonstrated better protective nature through reducing different free radicals. Lupeol the compound isolated from this plant extract was previously reported to possess different pharmacological activities like antiinflammatory, antiprotozoal, antimicrobial. Qualitative analysis of Z. armatum extracts in this investigation revealed the presence of phenolics, alkaloids, terpenoids and glycosides, which were reported to exert a variety of biological activities. Attempts to isolated pure components from the Z. armatum extract resulted in the isolation of lupeol, which was biologically active. Phenolic compounds, alkaloids and terpenoids could also have been responsible for the observed hepatoprotective activity as the Z. armatum extract had more phenolic and alkaloid content and also simultaneously exerted greater antioxidant and hepatoprotective activities. Previous studies reported that extracts with more phenolic contents possessed more antioxidant activity and hepatoprotective activity (Talluri et al., 2018).

2.20 Animal models for diabetes

No single animal model is entirely typical of diabetes in man and it's valuable to consider the lessons learned from a range of animal models that closely resemble various aspects of human diabetes (Baily and Flatt, 1986). Animal models of diabetes can be conveniently divided into those which are experimentally induced and those occur spontaneously.

The main experimentally induced models of diabetes are produced by surgical or chemical reduction of β-cell mass. STZ, N-methylurea derivative of 2-deoxy-D-glucose, is a β-cell toxin that selectively destroys β-cell. Alloxen (2, 4, 5, 6-tetraoxypyrimidine; 2, 4, 5, 6-pyrimedinetetrone) also selectively destroys β-cell of the islets of Langerhans.

Most of the spontaneously diabetic models exhibit obesity, hyperglycemia, islet β-cell hyperplasia hyperinsulinaemia and resistance all of which may become severe (Bailey and Flatt, 1990). The autosomal recessive obesity (diabetes syndromes of the ob/ob mouse) include all of these features and serve as a model of spontaneously occurring non-ketotic hyperinsulinemic type diabetes.

As for biological test system, most of the cases rats, mice, rabbits or guinea pigs are used in the laboratory. In case of animal model for diabetes, STZ (STZ), alloxan etc are injected

intraperitoneally to make the animal as a diabetic subject. For several decades, the β-cell specific toxin STZ (STZ) has been used to create animal models of diabetes, despite an incomplete understanding of how STZ actually causes β-cell death. For several decades, a dose of 50±100 mg of STZ, administered to a rat has been known to cause β-cell death within 8 hours and the subsequent development of diabetes. Diabetes simulating IDDM can be induced by intra peritoneal injection of STZ (60 mg/kg body weight) to adult (age 3-4 months) rats. NIDDM can be induced by intraperitoneal injection of STZ (90mg/kg body weight) to 48 hours pups (Bonner *et al.*, 1981).

2.21 Mechanism of STZ induced T2DM

Animal model of diabetes can be produced by use STZ. It's widely used to induce experimental diabetes due to its ability to selectively target and destroy insulin producing pancreatic-β cells.

STZ is a glucosamine-nitrosourea compound. As with other alkylating agents in the nitrosourea class, it is toxic to cells by causing damage to the DNA. It causes the fragmentation of DNA (Yamamoto *et al.*, 1981) in pancreatic islets and stimulates nuclear poly (ADP ribose) synthetase and thus depletes the intracellular NAD and NADP. NAD depletion by STZ inhibits proinsulin synthesis and thus induces diabetes (Oberley *et al.*, 1988).

STZ at low dose, damages pancreatic β cells by eliciting non-specific islet inflammation with infiltration by mononuclear cells (Like *et al.*, 1976). In addition, NO, generated by STZ has been proposed to be involved in the damage of pancreatic β cells(fig 2.2).

STZ is similar enough to glucose to be transported into the cell by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to β cells, since these cells have relatively high levels of GLUT2. It's an agent widely employed to induce experimental diabetes due to its ability to selectively target and destroy insulin producing pancreatic islet β cells (Johansson *et al.*, 1969; Gonzalez *et al.*, 2000).

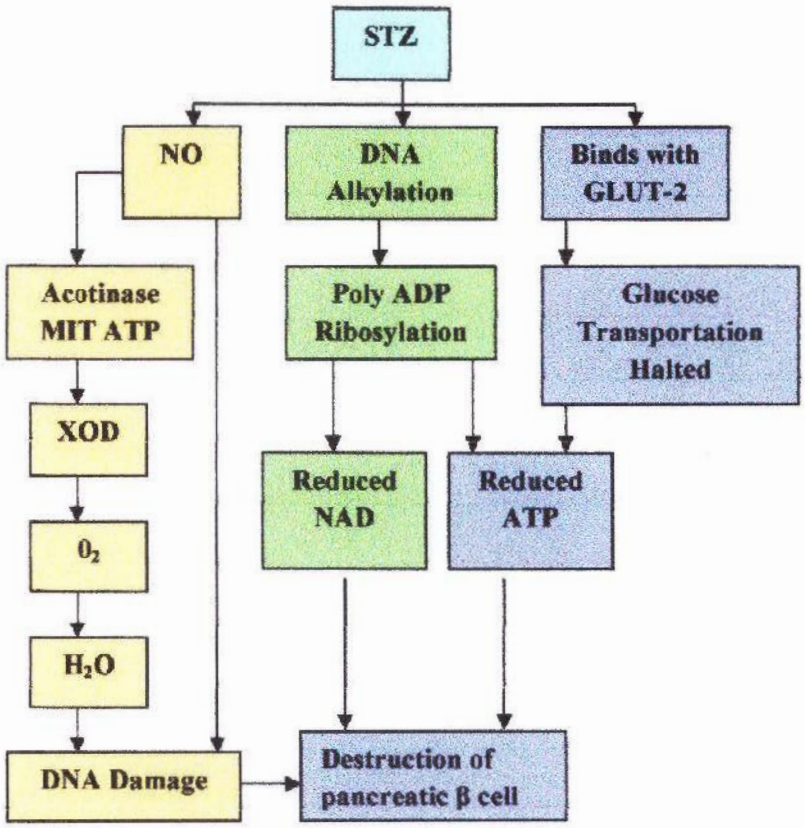

Figure 2.2: Proposed mechanism of action of STZ on β cell.

CHAPTER THREE: MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Place of the Study and Study Period

The study was conducted in the department of Pharmacology, Bangladesh University of Heath Sciences (BUHS), Dhaka, Bangladesh. The study was done during the period of July 2019 to November 2019.

3.2 Sample collection

5 kg fruits (Fig 3.1) of *Zanthoxylum armatum* were collected from hilly area of Kathmandu, Nepal. Then the fruits were dried at first sun light for 7 days and were more dried by Oven at 70^0C for 48 hours. For making powder the dried (Fig 3.2) fruits (2.5 Kg) were grinded by Grinding Machine (Fig 3.3) and obtained 2 kg of seeds powder.

Fig 3.1: Fruits of *Zanthoxylum armatum*

Fig 3.2: Dried fruits of *Zanthoxylum armatum*

Fig 3.3: Grinding of dried fruits of *Zanthoxylum armatum*

3.3 Preparation of methanolic extracts of *Zanthoxylum armatum* fruits

For the preparation of aqueous 80% methanol extract about 2 kg of the grinded powder was taken and extracted with 6L aqueous 80% methanol in three times (Fig 3.4) and left for 24 hours. The crude extract was separated and filtered (Fig 3.5) by using filter paper (What man No 1). Obtaining filtrate part (Fig 3.6) was then concentrated by using Rota-evaporator(Fig 3.7) in 40^0C and finally dried by using Freeze dryer (Fig 3.8) to obtain 88 g dry extract (oily black colored extract). The aqueous 80% methanol extract of *Zanthoxylum armatum* was stored in an air tight bottle kept in a refrigerator at 20^0C for evaluating further experiments.

Fig 3.4: Methanol with grinded powder of *Z. armatum*

Fig 3.5: Filtration process

Fig 3.6: Filtrate part separation

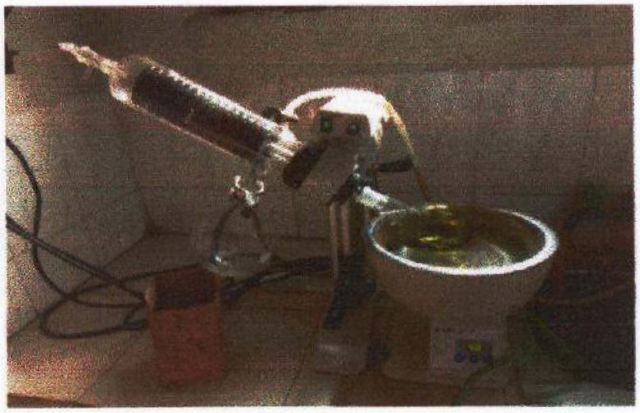

Fig3.7: Rota-evaporator

Fig 3.8: Freeze dryer

3.4 Animals

Thirty adult Long Evans rats weighing 190-270 g were included in the study. The animals were bred at Bangladesh University of Heath Sciences (BUHS) animal house, Dhaka, Bangladesh. Rats were maintained at a constant room temperature of 22±5^{0}C with humidity of 40-70 % and the natural 12 hours day-night cycle. The rats were fed on a standard laboratory pellet diet and water was supplied *ad libitum*. Standard rat pellet contained wheat (40%), wheat bran (20%), rice polishings (5%), fish meal (10%), oil cake (10%), gram (3.9%), pulses (3.9%), milk (3.8%), soyabean oil (1.5%), molasses (0.95%) and salt (0.95%). Embavit GS (vitamin mixture) 250g was added per 100kg of rat food.

3.5 Preparation of type 2 diabetic model rats

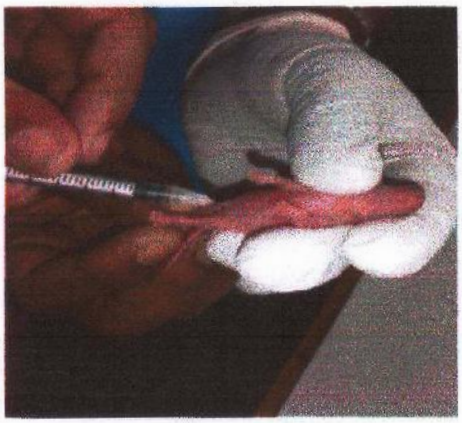

Fig 3.9.Injection of Streptozotocin for Type 2 model.

Type 2 diabetes was induced by a single intraperitoneal injection (Fig 3.9) of streptozotocin (STZ) in citrate buffer (10ml), at a dose of 90 mg/kg of body weight into the rat pups (48 hours old, average weight 7 gm) as described by Bonner-Weir et.al., (1981). Following 3 months of STZ injection, rats were examined for their blood glucose level by oral glucose tolerance test (OGTT). Diabetic model rats with blood glucose level >7.00 mmol/l, at fasting condition were selected for studying the effects of probiotic yoghurt.

3.6Formulation of gliclazide and *Zanthoxylum armatum* fruits methanolic extract(ZAFME) doses

For all the pharmacological studies, the drug gliclazide was prepared at a dose of 20 mg/5 ml/ kg body weight of Type 2 model rats. The *Zanthoxylum armatum* fruits methanolic extract(ZAFME) were given at 25mg, 50mg respectively.

3.7 Dose and route of administration

For the evaluation of the anti-diabetic activity, the *Zanthoxylum armatum* fruits methanolic extract(ZAFME) were administrated orally to the rats for 28 days at doses of 25mg, 50mg respectively in 2 groups. For all the pharmacological studies, the drug gliclazide administrated orally at a dose of 20 mg/5 ml/ kg body weight of Type 2 model rats. For the control groups, 10ml water was administrated per kg body weight.

3.8 Experimental design

Six normal rats and 30 type 2 diabetic model rats, total 36 long evans rats were used in this 21 days experimental period. They were divided into 6 groups shown in Table 3.1.

Table 3.1.Experimental rat groups

Group-1 (n=6) Standard(Std)	Normal water control rat
Group-2 (n=6) Negative Control(NC)	Diabetic water control rat [10 ml water/kg body weight]
Group-3 (n=6) Positive Control(NC)	Gliclazide treated diabetic rat 20 mg/5 ml/ kg body weight of Type 2 model rats
Group-4 (n=6) Treatment Group 1(TG 1)	25 mg/10 ml/ kg body weight of *Zanthoxylum armatum* fruits methanolic extract(ZAFME) treated rat
Group-5 (n=6) Treatment Group 2 (TG 2)	50 mg/10 ml/ kg body weight of *Zanthoxylum armatum* fruits methanolic extract(ZAFME) treated rat

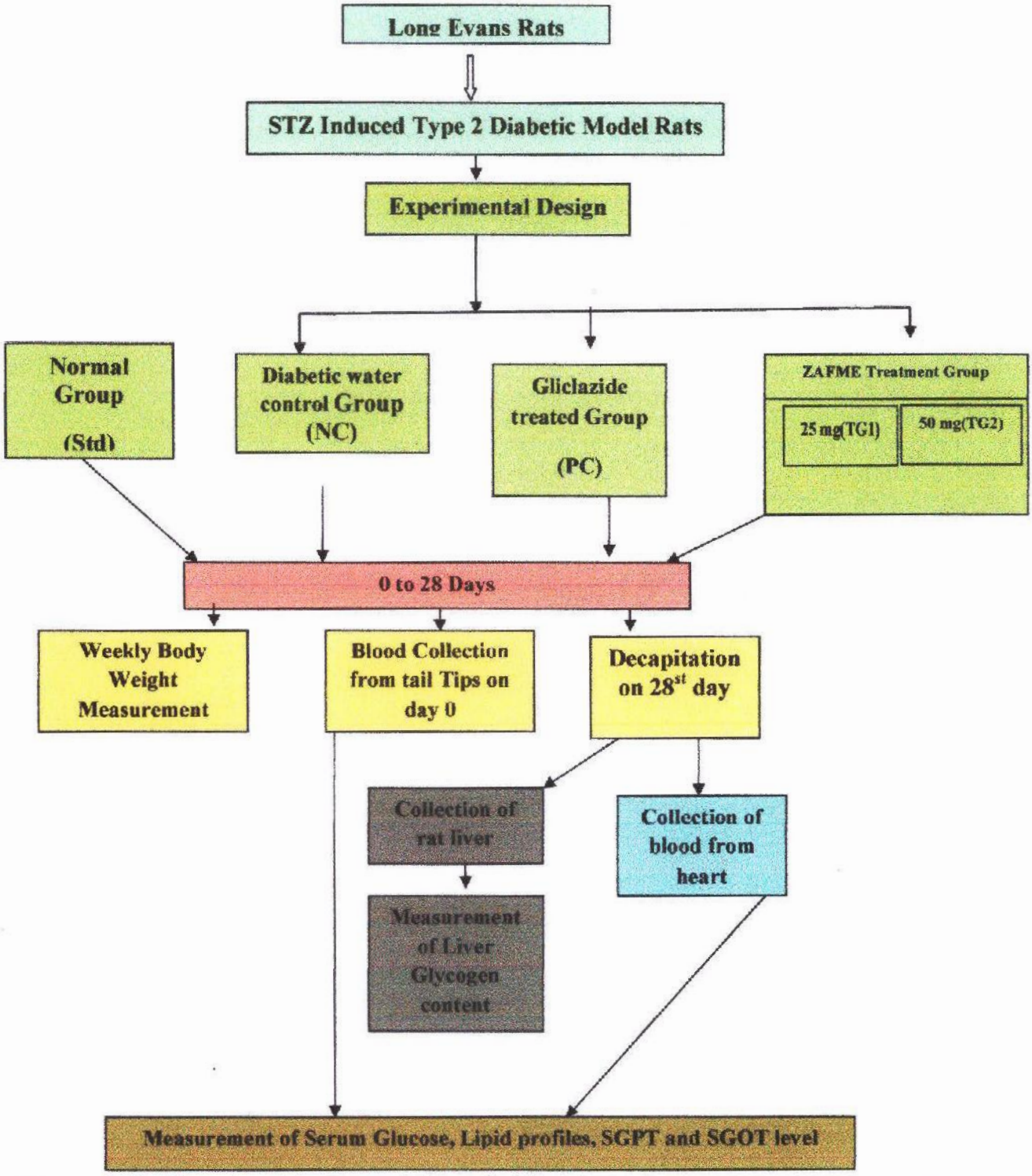

Figure 3.10 :Experimental protocol for administration of *Zanthoxylum armatum* fruits methanolic extract(ZAFME).

3.9 Collection of blood sample for biochemical analysis

Figure 3.11 : Blood collected from tail tip after amputation.

Blood samples were collected(Fig 3.11) from rats kept under fasting conditions by amputation of the tail tip under diethyl ether anesthesia at 0^{th} day. Just before the amputation, the tail was immersed into warm water (about 40°C) for approximately 20 - 30 seconds for vasodilatation. After cutting the tail tip, about 0.2 ml blood was taken cautiously in eppendorf tube to avoid haemolysis. On the 21^{th} day, after the animals were decapitated, their blood was collected from heart.

The collected blood samples were centrifuged at 2500 rpm for 15 minutes and finally the serums were separated(Fig 3.12) into another eppendorf tubes for biochemical analysis and 100µl of serum were kept frozen at -20°C until analysis of fasting serum insulin.

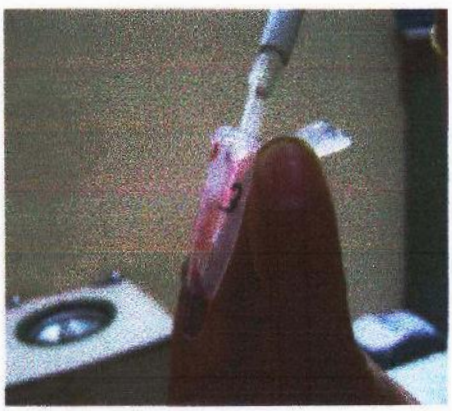

Figure 3.12: Separation of serum after centrifugation from the blood.

3.10 Recording of body weight

All the rats were ensured constant environmental condition and were provided with enough food and water throughout the experiment. The body weights of each rat was measured at seven days interval and accordingly they were provided with their respective treatment.

3.11 Biochemical analysis

The following parameters of type 2 diabetic model rats were measured for the anti-diabetic and antioxidant effects of probiotic yoghurt.

1. Serum glucose was measured by Glucose Oxidase (GOD-PAP) method using micro-plate reader (Bio-Tec, ELISA).

2. Serum total cholesterol by enzymatic colorimetric (Cholesterol Peroxidase/ Oxidase, CHOD-PAP) method (Randox Laboratories Ltd., UK), using autoanalyzer, AutoLab.

3.Serum triglyceride (TG) by enzymatic colorimetric (GPO-PAP) method (Randox Laboratories Ltd., UK) using autoanalyzer, AutoLab.

4. Measurement of glycogen from rat liver (standard Method).

5.SGPT and SGOT level is measured by enzyme kinetic method.

3.11.1 Serum glucose estimation by glucose oxidase (GOD-PAP) method (Boehringer-Mannheim Gmbh)

Principle: The Aldehyde group of β-D-glucose is oxidized by glucose oxidase to produce gluconic acid and hydrogen peroxide. Hydrogen peroxide is further broken down to water and oxygen in the presence of peroxidase and in presence of an oxygen acceptor (i.e. phenol) to produce a colored compound. The reaction of GOD-POD reagent with glucose produces 4-aminophenazone, a red colored compound (Barham and Trinder, 1972).

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone- mono-imino) phenazone} + 4H_2O$$

Reagents composition

a. Buffer : Phosphate buffer (0.1 mol/l, ph 7.0),
Phenol (11mol/l)

b. GOD-POD Reagent : 4-aminophenazone (0.77 mmol/l),
Glucose oxidase (≥ 1.5 kU/l)
Peroxidase (≥1.5 kU/l)

c. Standard Glucose (5.55 mmol/l).

Procedure:

While pipetting into the wells, the first two wells were kept blank and the 7 standard glucose solutions (5µl) of each concentration were pipetted in the next 7 wells of the micro-plate with duplicates. The serum samples (5µl) were pipetted(Fig 3.13) in the remaining micro-wells of the plate, each of them were pipeted twice. GOD-PAP(Fig 3.14) reagent (250µl) was then added in all the wells. The plate was then incubated in Lab systems iEMS Shaker incubator(Fig 3.15) for proper dilution for 15minutes at 37°C and using the Ultra micro-plate ELISA Reader (Bio-TekELx 808, USA), the absorbance of the samples were read at 512nm(Fig 3.16).

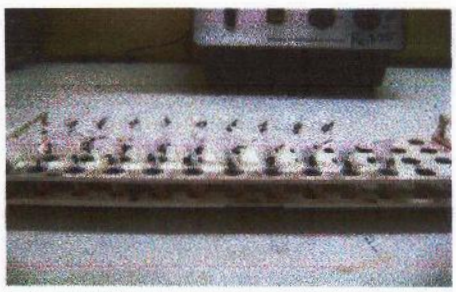

Figure 3.13:Serum samples of rats

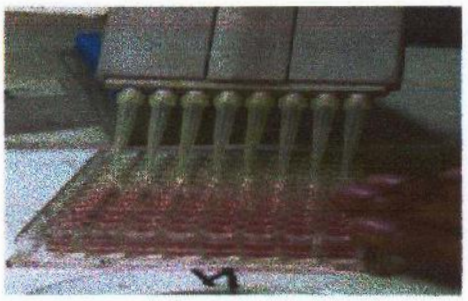

Figure 3.14:GOD-PAP reagent being added to all the samples for determination of glucose.

Figure 3.15: Lab system iEMS incubator.

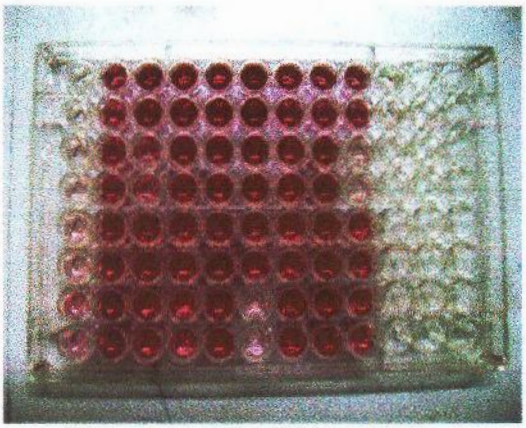

Figure 3.16: Microtiter plate with samples.

Two parallel experiments were carried out for each sample. Thus, a calibration curve was obtained for the absorbance vs. concentration of the standard solutions against a reagent blank. Based on the calibration curve(Fig 3.17), the unknown concentrations of glucose in the serum sample were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn parallel on every experiment day.

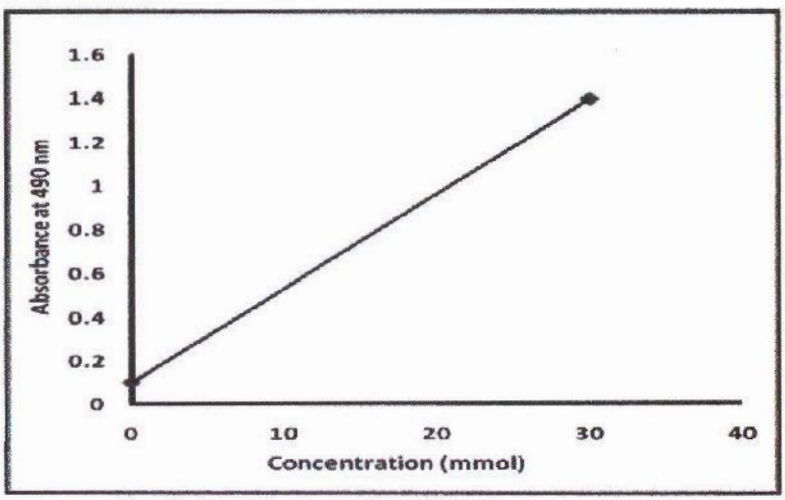

Figure 3.17: Standard curves for measuring glucose concentration

Calculation

In this GOD-PAP method (Boehringer-Mannheim GmbH), the concentration was calculated by using the kinetic calculation program for Ultra microplate ELISA(Fig 3.18) reader (Bio-Tek Elx-808, USA).

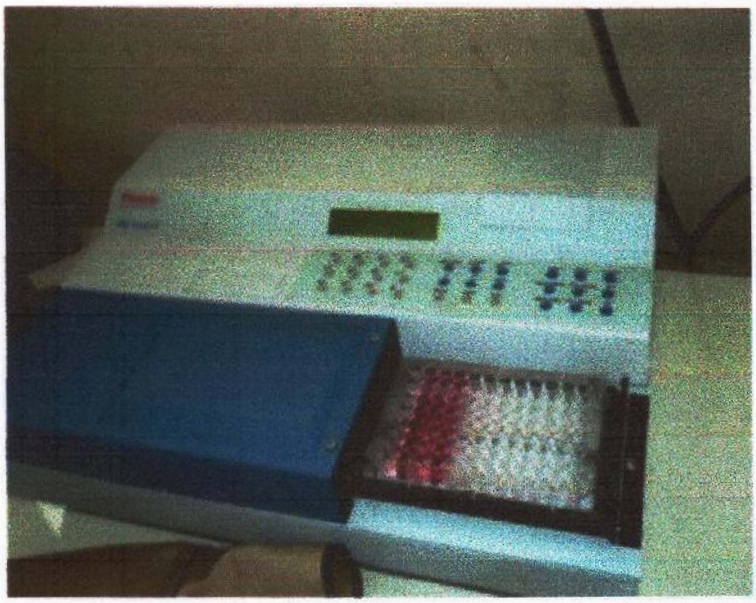

Figure 3.18: Ultra microplate ELISA reader

Calculation of result for unknown sample was as follows

$$\text{Concentrationof unknown sample} = \frac{\text{Optical density of unknown sample}}{\text{Optical density of standard}} \times \text{Standard Conc.}$$

$$\text{Glucose concentration (mmol/ l)} = \frac{A_{sample}}{A_{standard}} \times 5.55$$

$$\text{Glucose concentration (mg/ dl)} = \frac{A_{sample}}{A_{standard}} \times 100$$

Normal values: Serum, 4.2-6.4 mmol/l or 75-115 mg/dl (Teuscher *et al.*, 1971)

3.11.2 Estimation of serum total cholesterol by enzymatic colorimetric (cholesterol oxidase / peroxidase) method

Principle:

The Cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase (Trinder, 1969).

$$\text{Cholesterol esters} + H_2O \longrightarrow \text{Cholesterol} + \text{Fatty acids.}$$

$$\text{Cholesterol} + O_2 \longrightarrow \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \longrightarrow \text{Quinoneimmine} + 4H_2O$$

Reagent Composition

All reagents purchased from RANDOX, UK for the determination of the levels of cholesterol in human and rat serum samples.

Content	Initial Concentration Of Solution
Reagent	
4-Aminoantipyrine	0.30mmol/l
Phenol	6mmol/l
Peroxidase	>0.5U/ml

Cholesterol esterase	>0.15U/ml
Cholesterol Oxidase	>0.1U/ml
Pipes Buffer	80mmol/l; pH 6.8
Standard	5.17mmol/l (200 mg/dl)

Materials

- Micro-centrifuge tube
- Micropipettes and pipettes
- Disposable tips
- Automatic Analyzer (Boehringer Mannheim, 704; HITACHI)

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Automatic Analyzer. 5 µl sample and 500 µl reagent were mixed and incubated at 37°C for 5 minutes within the Auto Analyzer. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration of cholesterol in sample was calculated by using software program with the following formula and expressed in mg/dl.

$$\text{Cholesterol concentration (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Concentration of standard}$$

3.11.3 Estimation of serum triglyceride (TG) by enzymatic colorimetric method

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of Randox Laboratories Ltd., UK.

Principle

Sample triglycerides incubated with a lipoprotein lipase liberate glycerol and fatty acids. Glycerol was converted to glycerol-3-phosphate by glycerol kinase and ATP.Glycerol-3-phosphate oxidase (GPO) oxidizes glycerol-3-phosphate into dihydroxy acetone phosphate and H_2O_2. In the presence of peroxidase, hydrogen peroxide oxidizes the chromogen-4-aminoantipyrine and 4-chlorophenol to a violet colored complex. The quinone formed is proportional to the amount of triglycerides present in the sample. The principle is based on the following reaction system (Fossati and Prencipe, 1982).

$$\text{Triglyceride} + H_2O \xrightarrow{\textit{Lipase}} \text{glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[\textit{Kinase}]{\textit{Glycerol}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow[\textit{Oxidase}]{\textit{Glycerol Phosphate}} \text{dihydroxy acetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4- chlorophenol} \xrightarrow{\textit{Peroxidase}} \text{Quinoneimine} + HCl + 4\,H_2O$$

Reagents

Content	Concentrations in the Test
Buffer	
Pipes Buffer	40 mmol/l, pH 7.6
4-choloro-phenol	5.5 mmol/l
Magnesium-ions	17.5 mmol/l

Enzyme Reagent	
4-aminophenazone	
ATP	1.0 mmol/l
Lipases	>150 U/ml
Glycerol-3-phosphate oxidase	1.5 U/ml
Peroxidase	0.5 U/ml
Standard	2.29 mmol/l (200 mg/dl)

Materials

- Micropipettes and pipettes
- Disposable tips
- Auto analyzer

Procedure

Serum and reagents were taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five (5) µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the cell. Reading was taken at 500 nm.

Calculation of result

Triglyceride concentration was calculated by following formula:

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{Sample}}{A_{S\,\text{tan}\,dard}} \times \text{Concentration of standard}$$

3.11.4 Measurement of glycogen from rat liver

Liver glycogen levels were estimated by Anthrone-sulphiuric acid method (Vries, 1954).

First step: Preparation of glucose standard solution

Second step: Preparation of anthrone solution (Glucose stock 0.009375 gm/50ml)

3.11.4.1 Preparation of serial dilution from glucose stock

Concentration of glucose	Glucose	Water
0.3375mg/100ml	200µl	5.8ml
0.75mg/100ml	400µl	5.6ml
1.5mg/100ml	800µl	5.2ml
3.0mg/100ml	1.6ml	4.4ml
4.5mg/100ml	2.4ml	3.6ml
6.0mg/100ml	3.2ml	2.8ml
6.0mg/100ml	4.0ml	2.0ml

3.11.4.2 Anthrone solution

100 mg anthrone was dissolved in 50ml concentrated sulfuric acid.

3.11.4.3 Procedure

i. After scarification of rats on 21th day (different groups) 200mg liver was taken from each rat.

ii. Then it was homogenized with 10ml 5% TCA (Tricyclic Acid). First 100µl of TCA was added and grinded very well. After that rest of the TCA was gradually added and grinded, and made it 10ml. The solution was filtered(Fig 3.19) with a filter paper.

iii. 1ml of the filtrate was taken in 2ml 10N KOH in a test tube (pyrex) and was mixed by shaking. For standard and blank 1ml of 5% TCA in 2ml 10N KOH was taken.

iv. Both sample and standard were kept in boiling water or paraffin(Fig 3.20) (100°C) for 1hour.

v. After that they were made cool by using ice-cold water to stop the reaction.

vi. In the next step 1ml glacial acetic acid was added in each [sample, standard, blank (2 blanks and 7 standards were used)] test tube and made each solution as 10ml with deionized water for sample and blank. For standard, glucose solution was added and then made each solution as 10ml. Shaking mixed all the solutions very well.

vii. 1ml of this solution was added in 2ml of anthrone slowly and was mixed by shaking carefully as it became hot. All the test tubes were kept on ice.

viii. Again the test tubes were kept in boiling water or paraffin exactly for 10min.

ix. The test tubes were made cool on ice-cold water to stop the reaction.

x. 200µl solutions from each tube were added in a micro-titer plate and took the absorbance at 650nm(Fig 3.21).

xi. Using liner-liner graph paper, a standard curve was created by plotting the absorbance (Y) of each Reference Standard against its corresponding concentration (X) in mg/100ml.

xii. The average absorbance was used of each serum sample to determine the corresponding glucose value by simple interpolation from this standard curve (multiply the value by the initial sample dilution, if necessary).

Figure 3.19: Homogenized rat liver is being filtered.

Figure 3.20: Sample and standard solution in the heated paraffin.

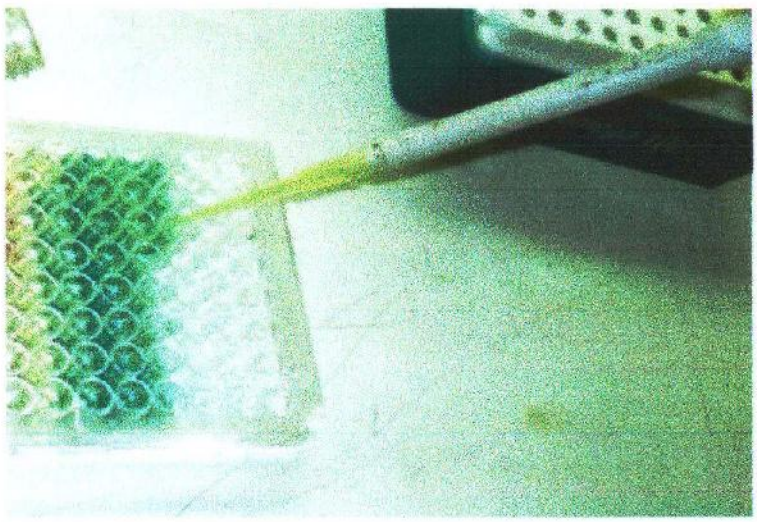

Figure 3.21: Micro-titer plate for measuring absorbance.

3.11.5.1 SGPT measurement through enzyme kinetic method

PRINCIPLE

$^{(\alpha}$-oxoglutarate + L-alanine $\xrightarrow{AST}$ L-glutamate + pyruvate

pyruvate + NADH + $H^+ \xrightarrow{LD}$ L-lactate+NAD^+

REAGENT COMPOSITON

Contents	Concentrations in the Test
R I a. Buffer/ Substrate	
Tris buffer	100 mmol/I. pH-7.5
L-alanine	0.6 mol/I
R I b. Enzyme/ Coenzyme/∝-oxoglutarate	
∝-oxoglutarate	156 mmol/I
LD	≥1.2U/ml
NADH	0.18 mmol/I

PROCEDURE

At first fresh ddH_2O is aspirated and performed a new Gain Calibration in flow cell mode. ALT in the Run Test Screen is selected and carry out a water blank as instructed(Fig 22).

Pipette into a test tube :

Sample	0.05 ml
Reagent	0.5 ml

Mix and aspirate into the Rx Monza.

FOR MANUAL USE

Wavelength	340 nm (Hg 334 nm or Hg 365 nm)
Cuvette :	I cm light path
Temperature :	30/37°C

Measurement : against air

Pipette into cuvette :

	Macro	Micro
Sample	0.2 ml	0.1 ml
R.I Enzyme/ Coenzyme/ $\propto$ –oxoglutarate	2.0 ml	1.0 ml

At first mixing, then reading is done by taking initial absorbance after 1 minute. Again reading is done after 1, 2 and 3 minutes. Note : If the absorbance change per minute is between :

0.11 and 0.16 at 340nm/Hg 334nm

0.06 and 0.08 at Hg 365 nm

only the values for the first 2 minutes is used for the calculation.

MANUAL CALCULATION

To Calculate the ALT activity, use the following formulae:

U/l = 1746 × Δ A 340 nm/min

U/l = 1780 × Δ A Hg 334 nm/min

U/l = 3235 × Δ A Hg 365 nm/min

Fig 22. SGPT & SGOT measurement through Autoanalyzer

3.11.5.1 SGOT measurement through enzyme kinetic method

PRINCIPLE

(α-oxoglutarate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The indicator reaction utilizes the oxaloacetate for a kinetic determination of NADH consumption.

(α-oxoglutarate + L-aspartate $\xrightarrow{AST}$ L-glutamate + oxaloacetate

oxaloacetate + NADH + $H^+ \xrightarrow{MDH}$ L-malate + NAD^+

REAGENT COMPOSITON

Contents	Concentrations in the Test
R I a. Buffer/ Substrate	
Tris buffer	80 mmol/l. pH-7.5
L-aspartate	240 mmol/l
R I b. Enzyme/ Coenzyme/α-oxoglutarate	
α-oxoglutarate	12 mmol/l
MDH	≥420U/l
LD	≥600U/l
NADH	0.18 mmol/l

PROCEDURE

At first fresh ddH_2O is aspirated and performed a new Gain Calibration in flow cell mode. Select AST in the Run Test Screen and carry out a water blank as instructed(Fig 22).

Pipette into a test tube :

Sample	0.05 ml
Reagent	0.5 ml

Mix and aspirate into the Rx Monza.

FOR MANUAL USE

Wavelength	340 nm (Hg 334 nm or Hg 365 nm)
Cuvette :	1 cm light path
Temperature :	25/30/37°C
Measurement :	*against air*

Pipette into cuvette :

	Macro	Micro
Sample	0.2 ml	0.1 ml
Enzyme/ Coenzyme/ $\propto$ –oxoglutarate R I	2.0 ml	1.0 ml

At first mixing, then reading is done by taking initial absorbance after 1 minute. Again reading is done after 1, 2 and 3 minutes. Note : If the absorbance change per minute is between :

0.11 and 0.16 at 340/Hg 334nm

0.06 and 0.08 at Hg 365 nm

only the values for the first 2 minutes is used for the calculation.

MANUAL CALCULATION

To Calculate the AST activity, use the following formulae:

U/I = 1746 × Δ A 340 nm/min

U/I = 1780 × Δ A Hg 334 nm/min

U/I = 3235 × Δ A Hg 365 nm/min

3.12 Statistical Analysis

Data from the experiments were analyzed using the Statistical Package for Social Science(SPSS) software 2016.

CHAPTER FOUR: RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1 Results

To generate a rat model mimicking human type 2 diabetes with impaired insulin secretion and insulin resistance, STZ injection (90 mg/kg, body weight) was induced into 48 hours old pulps. STZ injection to neonates led to the injury of the pancreas resulting in destruction of the functional β-cells. At the age of 3 months, when an oral glucose challenge (500mg/kg, body weight)was done, the remaining β cell could not cope with the load, which reflected in the postprandial rise of serum glucose level at 30 minutes (Table 4.1). The rise was significant among all rats compared to baseline value. Rats were selected and divided into different experimental groups to carry out the experiment.

Table 4.1. Oral glucose tolerance test in different groups of type 2 diabetic model rats considering fasting and postprandial serum glucose levels before the starting of experiment.

Group	OGTT	
	Fasting serum glucose level (mmol/L)	Postprandial serum glucose level (mmol/L)
NC(n=6)	6.98±1.73	14.31±5.40
PC(n=6)	7.27±1.60	14.31±4.48
TG1(n=6)	8.88±2.16	13.42±2.59
TG2(n=6)	7.19±1.34	14.76±4.44
One Way ANOVA		
NC Vs PC	1.000	1.000
NC Vs TG1	0.501	1.000
NC Vs TG2	1.000	1.000
PC Vs TG1	0.821	1.000
PC Vs TG2	1.000	1.000

TG1Vs.TG2	0.718	1.000
Paired sample t test (Fasting Vs Postprandial)		
NC(n=6)	0.007	
PC(n=6)	0.004	
TG1(n=6)	0.006	
TG2(n=6)	0.004	

Group NC, PC, TG1, TG2, represent diabetic water control rat, Gliclazide treated diabetic rat, 25mg *Zanthoxylum armatum* fruits methanolic extract treated rat, 50mg *Zanthoxylum armatum* fruits methanolic extract treated rats respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

4.1.1 Effects of *Zanthoxylum armatum* fruits methanolic extract on the body weight of type 2 diabetic model rats

A change in body weight in different group of rats was shown in depicted in Table 4.2. Initial body weight were 191g, 192g, 270g and 191g in NC, PC, TG1, TG2 groups respectively. As the body weight of rats under the study was taken every week; the changes has been reflected accordingly. After 28 days, the body weight of the respected groups was 206g, 206 g, 284 g and 208g. Therefore it was observed that body weight of all groups did increase.

Table 4.2: Effects of *Zanthoxylum armatum* fruits methanolic extract on the body weight of type 2 diabetic model rats

Group	Body weight (gm)				
	0 day	7 days	14 days	21 days	28 days
Std (n=6)	201±7.4 (100%)	200±5.1	209±0.5	208±6.5	215±3.0 (106%)
NC(n=6)	191±16 (100%)	188±19.2	191±16.5	196±27.0	206±28.4 (107%)
PC(n=6)	192±16.9	192±15.3	190±13.0	195±27.2	206±26.4

	(100%)				(107%)
TG1(n=6)	270±20 (100%)	271±24	288±24	281±26	284±25 (105%)
TG2(n=6)	191±19.8 (100%)	198±22.2	196±33.2	201±23.9	208±31.9 (108%)
One Way ANOVA					
Std Vs NC	1.000	1.000	1.000	1.000	1.000
Std Vs PC	1.000	1.000	1.000	1.000	1.000
Std Vs TG1	0.000	0.000	0.000	0.000	0.000
Std Vs TG2	1.000	1.000	1.000	1.000	1.000
NC Vs PC	1.000	1.000	1.000	1.000	1.000
NC Vs TG1	0.000	0.000	0.000	0.000	0.000
NC Vs TG2	1.000	1.000	1.000	1.000	1.000
PC Vs TG1	0.000	0.000	0.000	0.000	0.000
PC Vs TG2	1.000	1.000	1.000	1.000	1.000
TG1 Vs TG2	0.000	0.000	0.000	0.000	0.000

Group NC, PC, TG1, TG2, represent diabetic water control rat, Gliclazide treated diabetic rat, 25mg *Zanthoxylum armatum* fruits methanolic extract treated rat, 50mg *Zanthoxylum armatum* fruits methanolic extract treated rats respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

4.1.2 Effect of *Zanthoxylum armatum* fruits methanolic extract on fasting serum glucose level of type 2 diabetic rats

Results of fasting serum glucose (FSG) levels of the studied rats at baseline (before onset of feeding i.e. 0 day) and after 28 day of feeding are presented in Table 4.3. At baseline FSG levels of NC, PC, TG1, TG2 were almost similar (8.17±2.70; 8.67±0.30;7.84±1.63 and 7.52±1.49 mmol/l respectively). As expected FSG level of normal rats (Std)was lower 6.44±0.43 mmol/l.

RESULTS AND DISCUSSION

After oral administration of respective treatments for 28 days of experimental period, FSG level of all the groups except Std. and NC were decreased. Percentage decrease was 20%, 20% and 27% in PC, TG1, TG2, respectively. There were significantly lower blood glucose values in both groups (TG1 & TG2) of ZAFME and gliclazide group (PC) at endpoint compared to baseline ($p<0.023$, $p<0.041$ and $p<0.003$, respectively). Moreover, at the end point ZAFME at 50mg dose(TG2) decreased blood glucose significantly ($p<0.021$) compared to Diabetic water control (NC) group.

Table 4.3: Effect of *Zanthoxylum armatum* fruits methanolic extract (ZAFME) on fasting serum glucose level of type 2 diabetic rats

Group	Fasting Serum Glucose Level (mmol/L)		
	0 day	21 day	28day
Std (n=6)	6.44±0.43 (100%)	6.70±0.44	6.74±1.06 (104%)
NC(n=6)	8.17±2.70 (100%)	8.15±1.01	8.32±1.89 (101%)
PC(n=6)	8.67±0.30 (100%)	7.04±0.78	6.95±1.20 (80%)
TG1(n=6)	7.84±1.63 (100%)	7.40±0.55	6.30±0.77 (80%)
TG2(n=6)	7.52±1.49 (100%)	6.82±0.66	5.54±0.42 (73%)
One Way ANOVA			
Std Vs NC	1.000	0.795	1.000
Std Vs PC	0.793	1.000	1.000
Std Vs TG1	1.000	1.000	1.000
Std Vs TG2	1.000	1.000	1.000
NC Vs PC	1.000	0.093	0.754
NC Vs TG1	1.000	1.000	0.326
NC Vs TG2	1.000	1.000	0.021

PC Vs TG1	1.000	1.000	1.000
PC Vs TG2	1.000	1.000	1.000
TG1 Vs TG2	1.000	1.000	1.000

Paired sample t test (0day Vs 28day)		
Group	0day Vs 21day	0day Vs 28day
Std (n=6)	0.431	0.625
NC(n=6)	0.754	0.905
PC(n=6)	0.007	0.003
TG1(n=6)	0.587	0.023
TG2(n=6)	0.146	0.041

Group NC, PC, TG1, TG2, represent diabetic water control rat, Gliclazide treated diabetic rat, 25mg *Zanthoxylum armatum* fruits methanolic extract treated rat, 50mg *Zanthoxylum armatum* fruits methanolic extract treated rats respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

4.1.3 Effects of *Zanthoxylum armatum* fruits methanolic extract (ZAFME) on Lipid Profiles of type-2 diabetic model rats

Chronic effect of *Zanthoxylum armatum* fruits methanolic extract(ZAFME) on lipid profiles is presented in Table 4.4. There were 8%, 50% and 20% reduction in Triglyceride(TG) level shown in PC, TG1, TG2 on day 28 respectively. There were 13%, 6% and 22% reduction in cholesterol level shown in PC, TG1, TG2 on day 28 respectively. There were 20%, 6% and 14% increase in HDL level shown in PC, TG1, TG2 on day 28 respectively. There were 40%, 32% and 16% reduction in LDL level shown in PC, TG1, TG2 on day 28 respectively.

Table 4.4: Effects of *Zanthoxylum armatum* fruits methanolic extract on Lipid Profiles of type-2 diabetic model rats

Group	TG		Cholesterol		HDL		LDL	
	0 day	28 day	0 day	28 day	0 day	28 day	0 day	28 day

Std (n=6)	68.68±5.36 (100%)	74.22±0.19 (108%)	59.60±8.23 (100%)	63.57±5.97 (106%)	27.05±3.32 (100%)	25.68±1.07 (94%)	17.19±2.40 (100%)	19.55±2. (115%)
NC (n=6)	70.30±4.11 (100%)	73.61±10.39 (103%)	55.70±8.76 (100%)	63.74±4.41 (114%)	28.04±5.67 (100%)	25.78±0.87 (91%)	13.17±3.61 (100%)	15.26±6. (115%)
PC (n=6)	75.98±3.13 (100%)	70.14±9.72 (92%)	72.27±6.37 (100%)	63.52±4.41 (87%)	27.58±2.00 (100%)	33.25±3.97 (120%)	23.80±2.84 (100%)	14.46±2. (60%)
TG1 (n=6)	170.22±2.92 (100%)	86.71±6.71 (50%)	62.75±4.99 (100%)	59.16±4.4 (94%)	21.52±1.21 (100%)	23.02±0.68 (106%)	15.33±1.66 (100%)	10.53±1. (68%)
TG2 (n=6)	112.91±6.82 (100%)	84.14±5.03 (74%)	83.97±2.60 (100%)	66.19±1.65 (78%)	26.33±3.18 (100%)	30.02±4.12 (114%)	29.11±2.72 (100%)	24.53±2. (84%)
One Way ANOVA								
Std Vs NC	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Std Vs PC	1.000	1.000	0.284	1.000	1.000	0.002	1.000	1.000
Std Vs TG1	0.000	1.000	1.000	1.000	0.437	1.000	1.000	1.000
Std Vs TG2	0.000	1.000	0.000	1.000	1.000	0.309	0.036	1.000
NC Vs PC	1.000	1.000	0.923	1.000	1.000	0.002	0.137	1.000
NC Vs TG1	0.000	1.000	1.000	1.000	0.152	1.000	1.000	1.000
NC Vs TG2	0.000	1.000	0.000	1.000	1.000	0.353	0.003	1.000
PC Vs TG1	0.000	1.000	1.000	1.000	0.250	0.000	0.819	1.000
PC Vs TG2	0.001	1.000	0.341	1.000	1.000	1.000	1.000	0.962

TG1 Vs TG2	0.000	1.000	0.013	1.000	0.888	0.014	0.014	0.203

Paired sample t test (0day Vs 28 day)

Group	0 day Vs 28 day	0 day Vs 28 day	0 day Vs 28 day	0 day Vs 28 day
Std (n=6)	0.053	0.174	0.416	0.041
NC(n=6)	0.061	0.009	0.353	0.956
PC(n=6)	0.221	0.051	0.046	0.004
TG1(n=6)	0.000	0.317	0.068	0.010
TG2(n=6)	0.001	0.000	0.025	0.004

Group NC, PC, TG1, TG2, represent diabetic water control rat, Gliclazide treated diabetic rat, 25mg *Zanthoxylum armatum* fruits methanolic extract treated rat, 50mg *Zanthoxylum armatum* fruits methanolic extract treated rats respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

4.1.4 Effect of *Zanthoxylum armatum* fruits methanolic extract on hepatic glycogen content of type 2 diabetic model rats

The effects of *Zanthoxylum armatum* fruits methanolic extract on hepatic glycogen content of type 2 diabetic model rats are presented in Table 4.5. There were 56%, 15% and 35% increase in glycogen level shown in PC,TG1,TG2 on day 28 respectively.

Table 4.5: Effect of *Zanthoxylum armatum* fruits methanolic extract on hepatic glycogen content of type 2 diabetic model rats

Group	Liver Glycogen
Std (n=6)	15.92±0.84
NC(n=6)	14.03±4.02(100%)
PC(n=6)	21.89±1.41(156%)
TG1(n=6)	16.17±1.13(115%)

TG2(n=6)	19.01±0.90(135%)
One Way ANOVA	
Std Vs NC	1.000
Std Vs PC	0.012
Std Vs TG1	1.000
Std Vs TG2	1.000
NC Vs PC	0.000
NC Vs TG1	1.000
NC Vs TG2	0.065
PC Vs TG1	0.029
PC Vs TG2	1.000
TG1 Vs TG2	1.000

Group NC, PC, TG1, TG2, represent diabetic water control rat, Gliclazide treated diabetic rat, 25mg *Zanthoxylum armatum* fruits methanolic extract treated rat, 50mg *Zanthoxylum armatum* fruits methanolic extract treated rats respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

4.1.5 Effect of *Zanthoxylum armatum* fruits methanolic extract on SGPT level of type 2 diabetic model rats

The effects of *Zanthoxylum armatum* fruits methanolic extract on SGPT level of type 2 diabetic model rats are presented in Table 4.6. There were 18%, 60%, 15%, 46% and 26% increase in SGPT level shown in Std, NC, PC, TG1, TG2 on day 28, respectively. However in PC, TG1, TG2 reduced the SGPT level on day 28 when it compared with NC.

Table 4.6 : Effect of *Zanthoxylum armatum* fruits methanolic extract on SGPT(IU/L) level on type 2 diabetic model rats.

Experimental rat group	SGPT from 0 day to 28th day (M±SD)	
	0 day	28th day
Std (n=6)	48±4 (100%)	57±2 (118%)
NC(n=6)	50±3 (100%)	80±3 (160%)

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PC(n=6)	53±6 (100%)	61±5 (115%)
TG1(n=6)	47±3 (100%)	69±1 (146%)
TG2(n=6)	67±4 (100%)	85±1 (126%)

Group	One Way ANOVA	
	0 day	28th day
Std Vs NC	1.000	0.000
Std Vs PC	0.703	0.482
Std Vs TG1	1.000	0.000
Std Vs TG2	0.000	0.000
NC Vs PC	1.000	0.000
NC Vs TG1	1.000	0.000
NC Vs TG2	0.000	0.079
PC Vs TG1	0.289	0.001
PC Vs TG2	0.000	0.000
TG1 Vs TG2	0.000	0.000

Group	Paired sample t-test
	SGPT from 0 day Vs 28th day
Std (n=6)	0.000
NC(n=6)	0.000
PC(n=6)	0.051
TG1(n=6)	0.000
TG2(n=6)	0.000

Group NC, PC, TG1, TG2, represent diabetic water control rat, Gliclazide treated diabetic rat, 25mg *Zanthoxylum armatum* fruits methanolic extract treated rat, 50mg *Zanthoxylum armatum* fruits methanolic extract treated rats respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

4.1.6 Effect of *Zanthoxylum armatum* fruits methanolic extract on SGOT level of type 2 diabetic model rats

The effects of *Zanthoxylum armatum* fruits methanolic extract on SGOT level of type 2 diabetic model rats are presented in Table 4.7. There were 10%, 50%, 45%, 28% and 36% increase in SGOT level shown in Std, NC, PC, TG1, TG2 on 28th day respectively. But in PC,TG1,TG2 reduced the SGPT level on day 28 when it compared with NC.

Table 4.7: Effect of *Zanthoxylum armatum* fruits methanolic extract on SGOT(IU/L) level of type 2 diabetic model rats.

Experimental groups of rats	SGOT from 0 day to 28th day (M±SD)	
	0 day	28 day
Std (n=6)	83±7 (100%)	92±3 (110%)
NC(n=6)	111±5 (100%)	167±9(150%)
PC(n=6)	106±4 (100%)	154±7 (145%)
TG1(n=6)	74±4 (100%)	95±4 (128%)
TG2(n=6)	101±0.6 (100%)	138±6 (136%)

Group	One way ANOVA	
	0 day	28th day
Std Vs NC	0.000	0.000
Std Vs PC	0.000	0.000
Std Vs TG1	0.030	1.000
Std Vs TG2	0.000	0.000
NC Vs PC	1.000	0.024
NC Vs TG1	0.000	0.000
NC Vs TG2	0.014	0.000
PC Vs TG1	0.000	0.000

PC Vs TG2	0.533	0.004
TG1 Vs TG2	0.000	0.000

Group	Paired sample t-test
	SGOT from 0 day Vs 28th day
Std (n=6)	0.013
NC(n=6)	0.000
PC(n=6)	0.000
TG1(n=6)	0.000
TG2(n=6)	0.000

Group NC, PC, TG1, TG2, represent diabetic water control rat, Gliclazide treated diabetic rat, 25mg *Zanthoxylum armatum* fruits methanolic extract treated rat, 50mg *Zanthoxylum armatum* fruits methanolic extract treated rats respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

4.2 Discussion

Diabetes mellitus is possibly the world's largest growing metabolic disorder and as the knowledge on the heterogeneity of this disorder is advanced, the need for more appropriate therapy increases (Bailey *et al.*, 1986). Oxidative stress, through the production of reactive oxygen species (ROS), has been proposed as the root cause underlying the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance and type 2 diabetes mellitus (T2DM). It has also been implicated in the progression of long-term diabetes complications; including microvascular and macrovascular dysfunction (Wright *et al*, 2006). The enormous costs of modern medicines indicate that alternative strategies are required for better management of diabetes. Traditional plant medicines are used throughout the world for the management of diabetic mellitus and a range of diabetic complications. The study of such medicines might offer a natural key to unlock a diabetologist's pharmacy for the future (Nalamolu *et al.*, 2006).

RESULTS AND DISCUSSION

The aim of the present study was to investigate the effect of *Zanthoxylum armatum* fruits methanolic extract on serum glucose, lipids, liver glycogen, SGPT and SGOT contents of type 2 diabetic model rats after 28 days administration. Type 2 diabetes was developed by injecting STZ (a pancreatic β cell toxin) to 48 hours old pups, which dissociates the β-cell into glucose and methylnitrogenase. The later alkaylates and modifies biomolecules, breakdown DNA and destroys β cell; and thereby causing diabetes (Lezen S 2008). Early injury of the β cells resulted in the partial recovery of β cell leading to type 2 diabetes as a result of insulin resistance in target tissues and impaired insulin secretion, accompanied by increased adiposity. When at the age of 3 months these rats have been checked with a glucose load all of them could not cope with the glucose load. Although their fasting glucose values were a bit higher (ranging from 6.98-7.19 mmol/L) indicating the presence of some β cells. Their postprandial glucose values were significantly higher which proved that these rats have developed type 2 diabetes. The results of the study demonstrate that 28 days feeding of the extract increases body weight of the rats. The results of the present study demonstrate that fasting serum glucose level have been changed by the plant extracts. ZAFME significantly lowered fasting serum glucose level in type 2 diabetic rats after 28 days feeding. The results of the present study are in consistent with other investigators (Slei et al., 2012; Karki et al., 2014; Alam et al., 2018).

It is now well established that persistent hyperglycemia is an important cardiovascular risk factors in type 2 diabetic patients (Polisso, 2003). The association of hyperglycemia with an alteration of lipid parameters presents a major risk for cardiovascular complications in diabetes. In addition to glycemic control, treatment of hyperlipidemia also results in significant micro and macrovascular diseases in individuals with type2 diabetes. Therefore, in the present study, the effects of ZAFME on lipid profiles were evaluated upto 28 days chronic experiment. Administration of 25 mg and 50 mg ZAFME lowered triglyceride level by 50% and 20% respectively on day 28 in comparison to baseline values. Administration of 25 mg and 50 mg ZAFME lowered cholesterol level by 6% and 22% respectively on day 28 in comparison to baseline values. Administration of 25 mg and 50 mg ZAFME increased HDL level by 6% and 14% respectively on day 28 in comparison to baseline values. Administration of 25 mg and 50

mg ZAFME lowered LDL level by 32% and 16% respectively on day 28 in comparison to baseline values. The obtained results signify that ZAFME extract has cardioprotective effect.

Liver glycogen level may be considered as the best marker for assessing hypoglycemic activity of any drug. This indicates that peripheral free glucose is being stored in the liver in the form of glycogen by increasing glycogenesis. Administration of 25 mg and 50 mg ZAFME increased LDL level by 15% and 35% respectively on day 28 in comparison to baseline values. Therefore, it may be assumed that the hypoglycemic activity of ZAFME in type 2 diabetic model rats may be at least partly due to increase the uptake of glucose for the formation of glycogen by enhanced glycogenesis.

The determination of enzyme levels viz. SGPT and SGOT are largely utilised because necrosis or membrane damage of hepatocyte causes the release of these enzymes into the circulation. Phenolic compounds, alkaloids and terpenoids could also have been responsible for the observed hepatoprotective activity as the ZAFME had more phenolic and alkaloid content and also simultaneously exerted greater antioxidant and hepatoprotective activities (Talluri et al., 2018). Administration of 25 mg and 50 mg ZAFME lowered SGPT level by 46% and 26% respectively on day 28 in comparison to baseline values. Administration of 25 mg and 50 mg ZAFME lowered SGOT level by 28% and 36% respectively on day 28 in comparison to baseline values. The results of the present study are consistent with other investigators (Alam et al., 2018; Verma and Khosa, 2012 and Ranawat et al., 2010).

4.3 CONCLUSION

The present study aimed to screen the effect of *Zanthoxylum armatum* fruits methanolic extract on blood glucose, lipid profile, liver glycogen and enzymes in neonatal streptozotocin (STZ) induced type-2 diabetic rats. It was observed that *Zanthoxylum armatum* fruits methanolic extract significantly ($p<0.05$) lowered blood glucose level, reduced Triglycerides level significantly ($p<0.05$), reduced total cholesterol level and LDL level, increased HDL level and Hepatic glycogen level. SGPT and SGOT values were also significantly ($p<0.05$) reduced in Treatment Group 1 compared to Negative Control group. Overall, *Zanthoxylum armatum* fruits methanolic extract will be a special natural drug to manage type 2 diabetes.

4.4 LIMITATIONS

1. Time duration was sort.
2. In the beginning of the present study the dose calculation was so tough.

4.5 RECOMMENDATIONS

1. Identification of specific component from crude extract of *Zanthoxylum armatum* through HPLC.

2. Clinical trial of *Zanthoxylum armatum* fruits methanolic extract.

3. Drug development from *Zanthoxylum armatum* fruits.

REFERENCES

REFERENCES

Abbasi, A. M., Khan, M. A., & Zafar, M. (2013). Ethno-medicinal assessment of some selected wild edible fruits and vegetables of Lesser-Himalayas, Pakistan. *Pak J Bot*, *45*(SI), 215-222.

Afroz, A., Alam, K., Ali, L., Karim, A., Alramadan, M. J., Habib, S. H., Magliano, D.J. & Billah, B. (2019). Type 2 diabetes mellitus in Bangladesh: a prevalence based cost-of-illness study. *BMC health services research*, *19*(1), 601.

Ahmad, A., Misra, L. N., & Gupta, M. M. (1993). Hydroxyalk-(4Z)-enoic acids and volatile components from the seeds of Zanthoxylum armatum. *Journal of Natural Products*, *56*(4), 456-460.

Alam, F., & us Saqib, Q. N. (2017). Evaluation of Zanthoxylum armatum Roxb for inávitro biological activities. *Journal of traditional and complementary medicine*, *7*(4), 515-518.

Alam, F., us Saqib, Q. N., & Ashraf, M. (2018). Zanthoxylum armatum DC extracts from fruit, bark and leaf induce hypolipidemic and hypoglycemic effects in mice-in vivo and in vitro study. *BMC complementary and alternative medicine*, *18*(1), 68.

Alarcon-Aguilara, F. J., Roman-Ramos, R., Perez-Gutierrez, S., Aguilar-Contreras, A., Contreras-Weber, C. C., & Flores-Saenz, J. L. (1998). Study of the anti-hyperglycemic effect of plants used as antidiabetics. *Journal of ethnopharmacology*, *61*(2), 101-110.

ALLOXAN, S. L. E. I. (2012). Effects of Zanthoxylum zanthoxyloides leaves on blood glucose, lipid profile and some liver enzymes in alloxan induced diabetic rats.

American Diabetes Association. (2019). Summary of Revisions: Standards of Medical Care in Diabetes—2019. *Diabetes Care*, *42*(Supplement 1), S4-S6.

Anonymous (1970). Medicinal Plants of Nepal. Bulletin of the Department of Medicinal Plants . Department of Forestry and Plant Research, Ministry of Forests and Soil Conservation, Government of Nepal,(3).

ANSAB (2011). Enhancing Livelihood and Reducing Poverty of Mountain People by Linking High Value Product and Services, Value Chain Development Project, Final Progress Report. ANSAB, Kathmandu.

Arshad, M., & Ahmad, M. (2004). Medico-botanical investigation of medicinally important plants from Galliyat areas, NWFP (Pakistan). *Ethnobotanical Leaflets*, *2004*(1), 6.

Bachwani, M., Shrivastava, B., Sharma, V., Khandewal, R., & Tomar, L. (2012). An update review on Zanthoxylum armatum DC. *American Journal of Pharmtech Research*, *2*(1), 274-286.

REFERENCES

Bailey CJ, Flatt PR, Kwasowski P, Powel CJ, Marks V. (1986) Immunoreactive gastric inhibitory polypeptide & K cell hyperplasia & obese hyperglycemic (ob/ob) mice fed high fat & High carbohydrate cafeteria diets. *Acta Endocrinologica.* 112: 224-229.

Bailey CJ, Day C. (1989) Traditional plant medicines as treatment for diabetes. Diabetes Care, 12: 553-564.

Bailey CJ, Flatt PR. (1990) Adrenoceptor-mediated control of glucose homeostasis in obese hyperglycaemic (ob/ob) mice. *Diabetes Res* 14(2):87-91.

Baily CJ, Flatt PR. (1986) Antidiabetic drugs, new developments. *Ind Biotech*, 6:139-142

Balami, N. P. (2004). Ethnomedicinal uses of plants among the Newar community of Pharping village of Kathmandu District, Nepal. *Tribhuvan University Journal*, *24*(1), 13-19.

Balfour, J. A., & McTavish, D. (1993). Acarbose. *Drugs*, *46*(6), 1025-1054.

Barham D, Trinder P. (1972 Feb). An improved colour reagent for the determination of blood glucose by the oxidase system. *Analyst.* 97(151):142-5.

Barkatullah, I. M., Muhammad, N., Rehman, I., Rehman, M., & Khan, A. (2013). Chemical composition and biological screening of essential oils of Zanthoxylum armatum DC leaves. *J Clin Toxicol*, *3*(5), 1-6.

Barkatullah, M. I., JELANI, G., & AHMAD, I. (2014). Leaf, stem bark and fruit anatomy of zanthoxylum armatum dc.(rutaceae). *Pak. J. Bot*, *46*(4), 1343-1349.

Batool, F. A. R. H. A. T., Sabir, S. M., Rocha, J. B. T., Shah, A. H., Saify, Z. S., & Ahmed, S. D. (2010). Evaluation of antioxidant and free radical scavenging activities of fruit extract from Zanthoxylum alatum: a commonly used spice from Pakistan. *Pak J Bot*, *42*(6), 4299-311.

Bharti, S., & Bhushan, B. (2015). Phytochemical and Pharmacological Activities of Zantoxylum armatum. DC.: An Overview. *RESEARCH JOURNAL OF PHARMACEUTICAL BIOLOGICAL AND CHEMICAL SCIENCES*, *6*(5), 1403-1409.

Bhatt, T. D., Dhungana, A., Joshi, J., Yadav, P., & Basyal, C. (2018). Variation in Chemical Composition of Essential Oil Extracted from the Fruits of Zanthoxylum armatum DC.(Timur) of Nepal. *JOURNAL OF PLANT RESOURCES*, *16*(1), 100.

Bhattacharya, S., & Zaman, K. (2009). Essential oil composition of fruits and leaves of Zanthoxylum nitidum grown in upper Assam region of India. *Pharmacognosy research*, *1*(3), 148.

Bhattacharya, S. A. N. J. I. B., Zaman, M. K., & Haldar, P. K. (2009). Antibacterial activity of stem bark and root of Indian Zanthoxylum nitidum. *Asian J Pharm Clin Res*, *2*(1), 30-34.

Bonner S, Trent DF, Honey RN and Weir GC. (1981) Responses of neonatal rat islets on streptozotocin limited beta cell regeneration and hyperglycemia. *Diabetes*: 30: 64-69.

REFERENCES

Brijwal, L., Aseesh, P., & Tamta, S. (2013). An overview on phytomedicinal approaches of Zanthoxylum armatum DC.: An important magical medicinal plant. *Journal of Medicinal Plants Research*, *7*(8), 366-370.

Ceylan, S., Azal, Ö., Taşlipinar, A., Türker, T., Açikel, C. H., & Gulec, M. (2009). Complementary and alternative medicine use among Turkish diabetes patients. *Complementary therapies in medicine*, *17*(2), 78-83.

Collier, E., Watkinson, A., Cleland, C. F., & Roth, J. (1987). Partial purification and characterization of an insulin-like material from spinach and Lemna gibba G3. *Journal of Biological chemistry*, *262*(13), 6238-6247.

Das, N. G., Nath, D. R., Baruah, I., Talukdar, P. K., & Das, S. C. (1999). Field evaluation of herbal mosquito repellents. *The Journal of communicable diseases*, *31*(4), 241-245.

Deepika D, Satish SK, Lalit S. (2013) Current updates on anti-diabetic therapy. Journal of Drug Delivery & Therapeutics, 3(6), 121-126.

Dikshit, A., Naqvi, A. A., & Husain, A. (1986). Schinus molle: a new source of natural fungitoxicant. *Appl. Environ. Microbiol.*, *51*(5), 1085-1088.

DoF (2014). Value chain and designing of timur of Panchase protected forest area. Department of Forest. Ministry of Forests and Soil Conservation. Government of Nepal.

DPR (1983). Jadibuti Parichaya Mala. Jadibuti Sankalan, Samrakchyan, Sambardahn Bidhi. Department of Plant Resources, Ministry of Forest and Soil Conservation, Government of Nepal, (1-5), 15.

DPR (2011a). Catalogue of Nepalese Flowering Plants-II. Dicotyledons (Ranunculaceae to Dipsaceae). Department of Plant Resources, Ministry of Forest and Soil Conservation. Government of Nepal.

DPR (2006). Nepal Ko Aarthik Bikaska Lagi Prathamikta Prapta Jadibutiharu. Department of Plant Resources, Ministry of Forest and Soil Conservation, Government of Nepal (in Nepali).

DPR (2007). Medicinal Plants of Nepal (Revised edition). Bulletin of Department of Medicinal Plants 28. Department of Plant Resources, Ministry of Forests and Soil Conservation, Government of Nepal.

DPR (2016). Medicinal Plants of Nepal (Revised Second Edition). Department of Plant Resources, Ministry of Forest and Soil Conservation, Government of Nepal.

Dube, S., Kumar, A., & Tripathi, S. C. (1990). Antifungal and insect-repellent activity of essential oil of Zanthoxylum alatum. *Annals of Botany*, *65*(4), 457-459.

REFERENCES

Eddouks, M., Lemhadri, A., & Michel, J. B. (2004). Caraway and caper: potential anti-hyperglycaemic plants in diabetic rats. *Journal of ethnopharmacology*, *94*(1), 143-148.

Fossati P, Prencipe L (1982). Serum triglyceride determined colourimatrically with an enzyme that produce hydrogen peroxide. *Clin Chem* 28: 2077.

Gafner, S. (2018). Scientific Journals Increasingly Skeptical of Antioxidant Research. In *HerbalEGram* (Vol. 15). American Botanical Council.

Gao, H., Huang, Y. N., Gao, B., Xu, P. Y., Inagaki, C., & Kawabata, J. (2008). α-Glucosidase inhibitory effect by the flower buds of Tussilago farfara L. *Food chemistry*, *106*(3), 1195-1201.

Gilani, S. N., Khan, A. U., & Gilani, A. H. (2010). Pharmacological basis for the medicinal use of Zanthoxylum armatum in gut, airways and cardiovascular disorders. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *24*(4), 553-558.

Goel, A. K., Kulshreshtha, D. K., Dubey, M. P., & Rajendran, S. M. (2002). Screening of Indian plants for biological activity*: Part XVI.

Gonzalez E, Rosello-Catafau J, Jawerbaum A, Sinner D, Pustovrh C, Vela J. (2000) Pancreatic nitric oxide and oxygen free radicals in the early stages of streptozotocin-induced diabetes mellitus in the rat. *Brazilian Journal of Medical and Biology Research;* 33:1335.42.

Grover, J. K., Yadav, S., & Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. *Journal of ethnopharmacology*, *81*(1), 81-100.

Guglielmini, G., & Cristoni, A. (2002). Zanthoxylum alatum extract inhibits skin sensitivity. *Cosmetics and toiletries*, *117*(7), 47-54.

Guleria, S., Tiku, A. K., Koul, A., Gupta, S., Singh, G., & Razdan, V. K. (2013). Antioxidant and antimicrobial properties of the essential oil and extracts of Zanthoxylum alatum grown in North-Western Himalaya. *The Scientific World Journal*, *2013*.

Guo, T., Deng, Y. X., Xie, H., Yao, C. Y., Cai, C. C., Pan, S. L., & Wang, Y. L. (2011). Antinociceptive and anti-inflammatory activities of ethyl acetate fraction from Zanthoxylum armatum in mice. *Fitoterapia*, *82*(3), 347-351.

Hedge, I. C., & Long, D. G. (1994). Andrew John Charles Grierson (24 iv 1929-11 ix 1990). *Edinburgh Journal of Botany*, *51*(2), iii-vii.

Hertog, W. H., & Wiersum, K. F. (2000). Timur (Zanthoxylum armatum) Production in Nepal. *Mountain Research and Development*, *20*(2), 136-146.

REFERENCES

Hsieh, H. M., Wu, W. M., & Hu, M. L. (2009). Soy isoflavones attenuate oxidative stress and improve parameters related to aging and Alzheimer's disease in C57BL/6J mice treated with D-galactose. *Food and Chemical Toxicology*, *47*(3), 625-632.

International Diabetes Federation. (2018). IDF Diabetes Atlas—8th edition.

Jadhav, R., & Puchchakayala, G. (2012). Hypoglycemic and antidiabetic activity of flavonoids: boswellic acid, ellagic acid, quercetin, rutin on streptozotocin-nicotinamide induced type 2 diabetic rats. *Group*, *1*, 100g.

Jain, N., Srivastava, S. K., Aggarwal, K. K., Ramesh, S., & Kumar, S. (2001). Essential oil composition of Zanthoxylum alatum seeds from northern India. *Flavour and Fragrance Journal*, *16*(6), 408-410.

Johansson EB, Tjalve K. (1969) Studies on the tissue-disposition and fate of [14C] streptozotocin with special reference to the pancreatic islets. *Acta Endocrinologica;* 89:339-47.

Joshi, A. R., & Edington, J. M. (1990). The use of medicinal plants by two village communities in the central development region of Nepal. *Economic Botany*, *44*(1), 71-83.

Joshi, A. R., & Joshi, K. (2000). Indigenous knowledge and uses of medicinal plants by local communities of the Kali Gandaki Watershed Area, Nepal. *Journal of Ethnopharmacology*, *73*(1-2), 175-183.

Joshi, K. (2004). Documentation of Medicinal plants and their indigenous uses in Likhu Sub-watershed, Nepal. *Journal of Non-Timber Forest Products*, *11*(2), 86-93.

Joshi, S., & Gyawali, A. (2012). Phytochemical and biological studies on Zanthoxylum armatum of Nepal. *Journal of Nepal Chemical Society*, *30*, 71-77.

Kala, C. P. (2005). Ethnomedicinal botany of the Apatani in the Eastern Himalayan region of India. *Journal of Ethnobiology and Ethnomedicine*, *1*(1), 11.

Kalia, N. K., Singh, B., & Sood, R. P. (1999). A new amide from Zanthoxylum armatum. *Journal of natural products*, *62*(2), 311-312.

Kanwal, R., Arshad, M., Bibi, Y., Asif, S., & Chaudhari, S. K. (2015). Evaluation of Ethnopharmacological and Antioxidant Potential of Zanthoxylum armatum DC. *Journal of Chemistry*, *2015*.

Karki, H., Upadhayay, K., Pal, H., & Singh, R. (2014). Antidiabetic potential of Zanthoxylum armatum bark extract on streptozotocin-induced diabetic rats. *International Journal of Green Pharmacy (IJGP)*, *8*(2).

Karmakar, I., Haldar, S., Chakraborty, M., Dewanjee, S., & Haldar, P. K. (2015). Antioxidant and cytotoxic activity of different extracts of Zanthoxylum alatum. *Free Rad. Antiox*, *5*(1), 21-8.

REFERENCES

Kayat, H. P., Gautam, S. D., & Jha, R. N. (2016). GC-MS analysis of hexane extract of Zanthoxylum armatum DC. fruits. *Journal of Pharmacognosy and Phytochemistry*, *5*(2), 58.

Kumar, S., & Müller, K. (1999). Inhibition of keratinocyte growth by different Nepalese Zanthoxylum species. *Phytotherapy Research*, *13*(3), 214-217.

Kumud, U., & Kumar, A. P. (2010). Concentration dependent antioxidant activity of Zanthoxylum armatum. *Journal of Pharmacy research*, *3*(7), 1581-1582.

Kunwar, R. M., Uprety, Y., Burlakoti, C., Chowdhary, C. L., & Bussmann, R. W. (2009). Indigenous use and ethnopharmacology of medicinal plants in far-west Nepal. *Ethnobotany research and applications*, *7*, 005-028.

Kunwar, R.M., Pokharel Y.R. (2012). Zanthoxylum armatum DC (timur, Toothache tree) seeds in changing climate. Abstract. In: Proceedings of the Sixth National Conference on Science and Technology. NAST. Kathmandu.

Kunwar, R. M., Mahat, L., Acharya, R. P., & Bussmann, R. W. (2013). Medicinal plants, traditional medicine, markets and management in far-west Nepal. *Journal of ethnobiology and ethnomedicine*, *9*(1), 24.

Kwon, H. W., Kim, S. I., Chang, K. S., Clark, J. M., & Ahn, Y. J. (2014). Enhanced repellency of binary mixtures of Zanthoxylum armatum seed oil, vanillin, and their aerosols to mosquitoes under laboratory and field conditions. *Journal of medical entomology*, *48*(1), 61-66.

L. Warjeet Singh. (2011). Traditional medicinal plants of Manipur as anti-diabetics. Journal of Medicinal Plants Research Vol. 5(5), pp. 677-687.

Lenzen S. (2008) The mechanism of alloxan and streptozotocin induced diabetes. Diabetologia. 52 (2): 216-26.

Li, H., Li, P., Zhu, L., Xie, M., Wu, Z., (2006). Studies on the chemical constituents of Zanthoxylum armatum DC. Zhongguo Yaofang. Chin. Pharm CSIR, 2005. A Dictionary of Indian Materials and Industrial Products-Raw Materials Series. Publications and Information Directorate, Council of Scientific and Industrial Research, New Delhi, India,(17), 1035–1037.

Like AA, Rosani A. (1976). Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science*. 193:415-417.

Liu, J., Jia, L., Kan, J., & Jin, C. H. (2013). In vitro and in vivo antioxidant activity of ethanolic extract of white button mushroom (Agaricus bisporus). *Food and chemical toxicology*, *51*, 310-316.

REFERENCES

Loria, P., Lonardo, A., & Anania, F. (2013). Liver and diabetes. A vicious circle. *Hepatology Research, 43*(1), 51-64.

Malla, B., Gauchan, D. P., & Chhetri, R. B. (2014). Medico-ethnobotanical investigations in Parbat district of Western Nepal. *Journal of Medicinal Plants Research, 8*(2), 95-108.

Manandhar, N. P. (1987). Traditional medicinal plants used by tribals of Lamjung District, Nepal. *International journal of crude Drug Research, 25*(4), 236-240.

Manandhar, N.P. (2002). Plants and People of Nepal. Timber Press, Inc., Oregon, USA.

Manandhar, N. P. (2002). *Plants and people of Nepal*. Timber press.

Manandhar, A., & Tiwari, R. D. (2005). Antifungal efficacy of Zanthoxylum oil against Bipolaris sorokiniana (Sacc.) Shoem. *Ecoprint: An International Journal of Ecology, 12*, 91-93.

Marles RJ, Farnsworth NR (1995). Antidiabetic plants and their active constituents. Phytomed., 2: 133-189.

Mehta, D. K., Bhandari, A., Satti, N. K., Singh, S., Das, R., Suri, K. A., & Sharma, S. N. (2011). Anti-Inflammatory Activity of Methanolic extract of fruit of Zanthoxylum armatum. *Inventi Rapid: Planta Activa*.

Musabayane, C. T., Bwititi, P. T., & Ojewole, J. A. O. (2006). Effects of oral administration of some herbal extracts on food consumption and blood glucose levels in normal and streptozotocin-treated diabetic rats. *Methods and Findings in Experimental and Clinical Pharmacology, 28*(4), 223-228.

Nair, K. N., & Nayar, M. P. (1997). Rutaceae, Flora of India (Malpighiaceae-Dichapetalaceae)(editors: PK Hajra, VJ Nair, P. Daniel) Botanical Survey of India: Calcutta.

Nalamolu RK and Nammi S. (2006) Antidiabetic and renoprotective effects of the chloroform extract of Terminalia chebula Retz. seeds in streptozotocin-induced diabetic rats, *BMC Complementary and Alternative Medicine, 6*:17.

Negi, J. S., Bisht, V. K., Bh, A. K., Singh, P., & Sundriyal, R. C. (2011). Chemical constituents and biological activities of the genus Zanthoxylum: a review. *African Journal of Pure and Applied Chemistry, 5*(12), 412-416.

Negi, J. S., Bisht, V. K., Bhandari, A. K., Bisht, R., & Kandari Negi, S. (2012). Major constituents, antioxidant and antibacterial activities of Zanthoxylum armatum DC. essential oil. *Iranian Journal of Pharmacology and Therapeutics, 11*(2), 68-0.

REFERENCES

Oberley LW. (1988). Free radicals and diabetes. *Free Radical Biology and Medicine*; 5:113.24.

Oggero, A. J. (2016). Comparative morphology and anatomy of the leaf and stem of species of Zanthoxylum (Rutaceae) from central Argentina. *Polibotanica*, (42), 121-136.

Parajuli, R. R., Tiwari, R. D., Chaudhary, R. P., & Gupta, V. N. (2005). Fungitoxicity of the essential oils of some aromatic plants of Manang against Alternaria brassicicola. *Scientific World*, *3*(3), 39-43.

Paudel, K., Bhatt, T. D., Adhikari, A. K., & Basyal, C. (2017). Composition Comparison of Essential Oils of Zanthoxylum armatum DC. by GC-MS. *JOURNAL OF PLANT RESOURCES*, *15*(1), 81.

Peng, Y. H., Zhang, Y., Zeng, D. Q., Chen, F. F., Zhong, H. Y., Li, Z. H., & Huang, Y. (2009). Bioactivity and chemical composition of essential oil from Zanthoxylum beecheyanum var. alatum leaves against Culex pipens quinquefasciatus (Diptera: Culicidae). *Ying yong sheng tai xue bao= The journal of applied ecology*, *20*(6), 1488-1494.

Prakash, B., Singh, P., Mishra, P. K., & Dubey, N. K. (2012). Safety assessment of Zanthoxylum alatum Roxb. essential oil, its antifungal, antiaflatoxin, antioxidant activity and efficacy as antimicrobial in preservation of Piper nigrum L. fruits. *International journal of food microbiology*, *153*(1-2), 183-191.

Rai, S. K., & Pokhrel, M. (2006). Ethnobotanical Survey of Medicinal and Aromatic Plants of Chandannath, Depalgaun and Garjangkot Village Development Committees (VDCs) of Jumla, Mid Western Nepal. *Bulletin of Department of Plant Resources*, (27), 16-20.

Rajasekaran S, Sivagnanam K, Narayanan V and Subramanian S. (2001) Hypoglycemic and hypolipidemic effects of Aloevera on experimental rabbits. Publication of Indian Association of Biomedical Scientists. 41-45.

Rajbhandari, M., Wegner, U., Jülich, M., Schoepke, T., & Mentel, R. (2001). Screening of Nepalese medicinal plants for antiviral activity. *Journal of Ethnopharmacology*, *74*(3), 251-255.

Rajbhandari, M., Mentel, R., Jha, P. K., Chaudhary, R. P., Bhattarai, S., Gewali, M. B., ... & Lindequist, U. (2009). Antiviral activity of some plants used in Nepalese traditional medicine. *Evidence-Based Complementary and Alternative Medicine*, *6*(4), 517-522.

Rajbhandari, K. R. (2015). National Herbarium (KATH) and Flora of Nepal. *Bulletin of Dept. of Plant Resources*, (37), 1-18.

Ranawat, L., Bhatt, J., & Patel, J. (2010). Hepatoprotective activity of ethanolic extracts of bark of Zanthoxylum armatum DC in CCl4 induced hepatic damage in rats. *Journal of ethnopharmacology*, *127*(3), 777-780.

REFERENCES

Rynjah, C. V., Devi, N. N., Khongthaw, N., Syiem, D., & Majaw, S. (2018). Evaluation of the antidiabetic property of aqueous leaves extract of Zanthoxylum armatum DC. using in vivo and in vitro approaches. *Journal of traditional and complementary medicine*, *8*(1), 134-140.

Sati, S. C., Sati, M. D., Raturi, R., Badoni, P. P., & Singh, H. (2011). A new flavonoidal glycoside from stem bark of Zanthoxylum armatum. *IJPI's J. Pharm. Herb. Form*, *1*(2), 29-32.

Sati, S. C., Sati, M. D., Raturi, R., Badoni, P., & Singh, H. (2011). Anti-inflammatory and antioxidant activities of Zanthoxylum armatum stem bark. *Global Journal of researches in engineering: J General Engineering*, *5*, 86.

Shah, N. C. (1991). Chemical composition of the pericarp oil of Zanthoxylum armatum DC. *Journal of Essential Oil Research*, *3*(6), 467-468.

Sharma, P. K., Raina, A. P., & Dureja, P. (2009). Evaluation of the antifungal and phytotoxic effects of various essential oils against Sclerotium rolfsii (Sacc) and Rhizoctonia bataticola (Taub). *Archives of Phytopathology and Plant Protection*, *42*(1), 65-72.

Shrestha, P. (1985). Research note: contribution to the ethnobotany of the Palpa area. *Contribution to*.

Shrestha, P. (1988). Contribution to the ethnobotany of the Tamangs of Kathmandu valley. *Contributions to Nepalese Studies*, *15*(2), 247-266.

Shruthi, A., Latha, K. P., Vagdevi, H. M., Pushpa, B., & Shwetha, C. (2012). Anti-diabetic activity of the leaves extracts of Wrightia Tinctoria on alloxan induced diabetic rats. *J Chem Pharm Res*, *4*(6), 3125-3128.

Singh, B., Uniyal, A. K., & Todaria, N. P. (2007). Studies on allelopathic influence of Zanthoxylum armatum DC on important field crops seeking its sustainable domestication in existing agroforestry systems of Garhwal Himalaya, India. *Journal of sustainable agriculture*, *30*(3), 87-95.

Singh, T. P., & Singh, O. M. (2011). Phytochemical and pharmacological profile of Zanthoxylum armatum DC.-an overview.

Singh, O. J., Raleng, I., Premchand, M., & Debashree, N. (2016). A review on the pharmacological profiles of Zanthoxylum armatum DC (Rutaceae). *J. Evol. Res. Med. Pharmacol.*, *2*(1), 10-12.

Subedi, R. (2017). *Ethnobotanical Study of Panchase Protected Forest, Kaski District, Central Nepal* (Doctoral dissertation, Central Department of Botany Tribhuvan University Kirtipur, Kathmandu, Nepal).

REFERENCES

Swanston SK, Day C, Bailey CJ, Flatt PR. (1990) Traditional plant treatments for diabetes Studies in normal and streptozotocin diabetic mice. Diabetologia, 33, 462-464

Talluri, M. R., Gummadi, V. P., Battu, G. R., & Killari, K. N. (2019). Evaluation of Hepatoprotective Activity of Zanthoxylum armatum on Paracetamol-induced Liver Toxicity in Rats. *Indian Journal of Pharmaceutical Sciences*, *81*(1).

Tara, J. S., Sudan, M. A. D. H. U., & Sharma, B. A. L. D. E. V. (2011). A report on the occurrence of insect pests on Zanthoxylum armatum DC (Family: Rutaceae), an important medicinal plant in jammu region. *The Bioscan*, *6*(2), 223-228.

Tiwary, M., Naik, S. N., Tewary, D. K., Mittal, P. K., & Yadav, S. (2007). Chemical composition and larvicidal activities of the essential oil of Zanthoxylum armatum DC (Rutaceae) against three mosquito vectors. *Journal of vector borne diseases*, *44*(3), 198.

Trinder P. (1969), *Ann clin Biochem* 6: 24.

Turin, M. (2003). Ethnobotanical notes on Thangmi plant names and their medicinal and ritual uses. *Contributions to Nepalese Studies*, *30*(1), 19-52.

Uprety, Y., Asselin, H., Boon, E. K., Yadav, S., & Shrestha, K. K. (2010). Indigenous use and bio-efficacy of medicinal plants in the Rasuwa District, Central Nepal. *Journal of Ethnobiology and Ethnomedicine*, *6*(1), 3.

Verma, N., & Khosa, R. L. (2012). *Hepatoprotective Effect of Zanthoxylum armatum DC* (p. 25). InTech.

Vries, J. V. (1954). Two methods for the determination of glycogen in liver. *Biochemical Journal* 57: 410-416.

Waheed, A., Mahmud, S., Akhtar, M., & Nazir, T. (2011). Studies on the Components of Essential Oil of Zanthoxylum armatum by GC-MS. *American Journal of Analytical Chemistry*, *2*(02), 258.

World Health Organization. (2016). *Global report on diabetes: executive summary* (No. WHO/NMH/NVI/16.3). World Health Organization.

World Health Organization. (2019). Classification of diabetes mellitus.

World Health Organization. (1998). *Obesity: preventing and managing the global epidemic: report of a WHO consultation on obesity, Geneva, 3-5 June 1997* (No. WHO/NUT/NCD/98.1). Geneva: World Health Organization.

Wright JR, Bacon JLS, and Glass LC. (2006 March) Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycaemia, *Int J Clin Pract.* ; 60(3): 308–314.

REFERENCES

Yamamoto H, Uchigata Y, Okamoto H. (1981) Streptozotocin and alloxan induce DNA strand breaks and alloxan and poly (ATPribose) synthetase in pancreatic islets. *Nature.* 294: 284-286.

Zhang, Y., Peng, Y., Zeng, D., Chen, F., Qin, Q., & Huang, Y. I. (2010). Insecticidal activity of essential oil from Zanthoxylum armatum fructification against two mosquito species. *Guangxi Zhiwu/Guihaia*, *30*(2), 274-279.

https://en.wikipedia.org/wiki/Liver

https://www.randox.com/alt/

https://www.randox.com/ast/

http://www.theplantlist.org/1.1/ browse/A/Rutaceae/Zanthoxylum/

APPENDIX

Appendices

Appendix Table 1. Weekly body weight data of all the experimental groups of rats.

Group	Rat No.	BW-0 day	BW-7 day	BW-14 day	BW-21 day	BW-28 day
Std	R 1	198	198	207	202	216
Std	R 2	202	203	210	213	213
Std	R 3	197	202	210	210	215
Std	R 4	202	197	207	206	213
Std	R 5	194	195	205	206	215
Std	R 6	199	197	207	200	215
NC	R 1	187	174	188	184	190
NC	R 2	190	185	185	179	194
NC	R 3	184	177	182	184	188
NC	R 4	197	207	209	210	219
NC	R 5	170	170	171	176	190
NC	R 6	219	217	214	246	260
PC	R 1	181	180	184	177	187
PC	R 2	192	190	191	190	206
PC	R 3	167	171	170	156	165
PC	R 4	214	212	208	228	237
PC	R 5	196	200	193	200	214
PC	R 6	206	203	199	222	227
TG1	R 1	271	273	288	280	280
TG1	R 2	263	254	280	269	273
TG1	R 3	240	240	252	247	253
TG1	R 4	293	297	315	318	320
TG1	R 5	286	294	308	295	297
TG1	R 6	286	294	308	295	297
TG2	R 1	174	166	158	168	173
TG2	R 2	191	200	188	200	198
TG2	R 3	174	196	188	200	199
TG2	R 4	180	184	170	185	187
TG2	R 5	213	222	239	235	254
TG2	R 6	219	224	234	220	242

Appendices

Appendix Table 2. OGTT & blood glucose data all the experimental groups of rats.

Group	Rat No	OGTT-0 min	Postprandial -90 min	Glu-0 day	Glu-21 day	Glu-28 day
Std	R 1			6.80	6.40	7.24
Std	R 2			6.42	5.98	6.80
Std	R 3			6.96	6.79	4.79
Std	R 4			6.63	6.87	6.72
Std	R 5			8.90	7.14	8.01
Std	R 6			8.98	7.07	6.92
NC	R 1	10.02	19.68	10.53		10.90
NC	R 2	6.42	9.80	12.51	8.15	7.67
NC	R 3	7.25	18.53	7.02	9.45	7.60
NC	R 4	5.81	8.68	6.13	8.26	6.80
NC	R 5	5.01	9.72	5.82	8.57	10.50
NC	R 6	7.41	19.45	7.01	6.33	6.50
PC	R 1	7.11	19.89	8.94	7.76	7.81
PC	R 2	5.80	10.20	8.67	7.04	6.11
PC	R 3	5.39	8.46	8.71	5.60	5.14
PC	R 4	9.83	18.44	8.67	7.04	6.63
PC	R 5	8.05	13.65	8.11	7.76	6.95
PC	R 6	7.44	15.25	8.94	7.04	7.16
TG1	R 1	10.53	16.08	7.84	7.40	6.09
TG1	R 2	8.18	9.89	8.18	6.48	6.94
TG1	R 3	10.36	13.94	7.84	7.59	6.30
TG1	R 4	5.38	11.73	5.38	7.59	5.14
TG1	R 5	9.96	15.49	9.96	7.94	7.06
TG1	R 6	9.96	15.49	9.96	7.94	7.06
TG2	R 1	7.94	22.15	6.68	6.89	5.57
TG2	R 2	7.35	14.87	7.52	6.11	4.85
TG2	R 3	6.26	9.95	6.63	6.11	5.54
TG2	R 4	5.08	10.35	6.63	7.13	5.54
TG2	R 5	8.89	15.29	6.52	5.57	5.54
TG2	R 6	7.63	15.97	5.80	4.85	6.20

Appendices

Appendix Table 3. Triglycerides & Cholesterol measurement data of all the experimental groups of rats.

Group	Rat No.	TG-0 day	TG-28 day	Chol-0 day	Chol-28 day
Std	R 1	74.54	74.23	67.07	61.61
Std	R 2	59.39	74.22	62.92	70.34
Std	R 3	65.70	74.22	46.97	52.87
Std	R 4	71.39	74.22	51.77	64.23
Std	R 5	70.54	74.54	65.95	66.63
Std	R 6	70.54	73.91	62.92	65.76
NC	R 1	71.78	77.70	62.92	63.74
NC	R 2	70.75	70.75	47.70	58.77
NC	R 3	65.00	72.77	42.72	57.89
NC	R 4	73.60	73.60	55.49	63.14
NC	R 5	75.00	76.11	61.82	69.03
NC	R 6	65.70	70.75	63.57	69.91
PC	R 1	74.75	69.00	65.10	63.35
PC	R 2	76.12	66.00	71.98	68.38
PC	R 3	73.28	60.00	71.98	68.60
PC	R 4	74.75	68.57	83.67	59.20
PC	R 5	82.12	68.57	73.19	63.52
PC	R 6	74.86	88.75	67.72	58.11
TG1	R 1	172.13	93.50	63.58	64.01
TG1	R 2	173.08	86.71	69.91	59.43
TG1	R 3	170.20	75.81	62.75	57.02
TG1	R 4	170.20	86.86	61.61	52.87
TG1	R 5	165.50	90.68	55.93	62.48
TG1	R 6	165.50	90.68	55.93	62.48
TG2	R 1	107.07	84.14	83.97	63.35
TG2	R 2	107.07		81.11	68.38
TG2	R 3	125.70	89.17	87.60	67.07
TG2	R 4	111.81	89.17	86.06	66.19
TG2	R 5	112.91	79.60	81.11	66.19
TG2	R 6	112.91	78.65	83.97	65.98

Appendices

Appendix Table 4. HDL, LDL & Glycogen measurement data of all the experimental groups of rats.

Group	Rat No.	HDL-0 day	HDL -28 day	LDL -0 day	LDL -28 day	Glyc-28 day
Std	R 1	26.20	26.61	18.77	18.68	15.38
Std	R 2	21.62	24.91	20.60	22.76	17.55
Std	R 3	29.38	25.44	17.32	18.68	15.9
Std	R 4	25.41	27.32	16.51	22.76	15.38
Std	R 5	30.67	24.44	16.51	18.68	15.38
Std	R 6	29.02	25.41	13.46	15.77	15.92
NC	R 1	31.61	25.87	15.77	13.46	15.08
NC	R 2	22.09	28.87	11.16		18.13
NC	R 3	22.21	26.14	7.76		10.39
NC	R 4	24.68	24.15	16.09	9.49	18.68
NC	R 5	33.37	26.79		13.18	13.15
NC	R 6	34.31	25.87	15.11	24.93	8.79
PC	R 1	26.50	37.37	23.80	11.11	20.05
PC	R 2	28.49	35.13	20.00	18.02	23.71
PC	R 3	24.68	36.90	26.19	17.04	23.02
PC	R 4	29.96	28.67	26.08	11.70	22.17
PC	R 5	26.56	28.32	26.08	14.46	20.50
PC	R 6	29.32	33.14	20.65	14.46	21.89
TG1	R 1	20.74	22.80	15.33	8.41	14.95
TG1	R 2	23.50	22.62	15.32	11.79	17.76
TG1	R 3	21.52	23.98	17.89	10.53	15.21
TG1	R 4	20.33	22.27	13.23	11.40	16.48
TG1	R 5	21.52	23.45	14.91	10.53	16.48
TG1	R 6	21.52	23.45	14.91	10.53	16.48
TG2	R 1	24.56	27.73	25.86	25.56	19.01
TG2	R 2	26.03	28.91	25.86	20.99	19.01
TG2	R 3	30.55	30.08	31.68	25.56	20.50
TG2	R 4	29.85	38.19	32.03	25.86	19.01
TG2	R 5	22.68	27.32	29.14	23.17	18.91
TG2	R 6	24.32	27.91	30.09	26.05	17.64