CHAPTER 4

To study the toxicity of microwave combined ultrasound treated *Lagenaria siceraria* juice and its antidiabetic and anti-inflammatory effects

4.1. Introduction

Plants have always played an important part in the healthcare systems of all the world's civilizations and cultures because of the widespread usage of products that are made from plants. The traditional uses of herbs as medicine are deeply ingrained in the cultures of most underdeveloped nations, where they also constitute the primary modality of therapeutic intervention. These treatments, which are successful to a significant degree, are well recognised by society, are economically feasible, and are, for the most part, the only source that is accessible [14]. Because of this, plants that are used in traditional medicine play an important role in the upkeep of people's health all over the globe. Since the beginning of time, those who practise traditional medicine have relied on medicines derived from plant, herbo-mineral, and animal sources to preserve health and cure illness. These kinds of treatments are used extensively throughout Asia and Africa, notably in India and China. In wealthy nations as well as developing ones, the use of medications produced from plants is becoming more common [6]. This is partly because synthetic pharmaceuticals often have undesirable side effects, and also because resistance to synthetic drugs can develop. Recent research, however, has revealed that several therapeutic herbs can also be harmful [11]. Because of this, there is cause for concern over the possibility of harmful effects coming from the prolonged use of therapeutic herbs. The evaluation of the toxicological effects of any medicinal plant extract that is intended for use in clinical or preclinical settings is, as a result, an essential component of the assessment of the possible hazardous consequences of the extract.

Diabetes mellitus is a metabolic illness that is characterised by unusually high amounts of sugar in the blood (World Health Organization, 2019). In addition to this, it is distinguished by a disturbance in the metabolism of carbohydrates, fats, and proteins as a result of a deficiency in insulin production and/or action on the target tissues. There are several subtypes of diabetes, each of which is linked to a unique set of causes, diagnostic signs, and therapeutic treatment strategies. However, there are two separate kinds of diabetes mellitus: immune-mediated type 1 diabetes (also known as T1DM), and metabolism-mediated type 2 diabetes (also known as T2DM) [9].

The present study investigates the antidiabetic and anti-inflammatory potential of *Lagenaria siceraria* juice. The study also investigates the toxicity of *Lagenaria siceraria*

juice. Though *Lagenaria siceraria* is a common vegetable and used in different cuisines but the use of the vegetable as a juice had to be checked for its toxicity.

4.2. Materials and methods

4.2.1. Toxicity study of lyophilized *Lagenaria siceraria* juice

4.2.1.1. Cell viability assay of lyophilized *Lagenaria siceraria* juice in (A) Human Erythrocytes (B) Human peripheral blood mononuclear cells (HPBMC) and (C) THP-1 human monocyte cell line

(A) Cell viability assay of lyophilized *Lagenaria siceraria* juice in Human Erythrocytes

Blood from humans was obtained with their consent. After isolating RBC from blood samples, they were washed three times in PBS with a pH of 7.4, and then they were centrifuged at 2000 rpm for ten minutes. The erythrocyte suspension (ES) at a concentration of 2% was re-suspended in saline solution. After adding the reaction mixture that consists of 100 µL of ES, 0.1% Triton X-100, and 25, 50, and 100 g/ml of lyophilized *Lagenaria siceraria* juice to 96-well microplates, the plates were then incubated at 37 °C for 60 minutes with continual agitation. After centrifugation at 2000 rpm for ten minutes, a photometric examination of the supernatant was performed at 576 nm [4] to measure the amount of haemoglobin that was released.

(B) Cell viability assay of lyophilized *Lagenaria siceraria* juice in Human peripheral blood mononuclear cells (HPBMC)

The cytotoxic effect of lyophilized *Lagenaria siceraria* juice was investigated in Human peripheral blood mononuclear cell (HPBMC) [4]. Isolated PBMC (3×10^3 cells in 200 µL) was seeded in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) in ninety-six well plates [5]. Histopaq buffer was used for separation of PBMCs. Firstly, cells were incubated without FBS at 37 °C in 5% CO_2 for 8 h. The cells were treated with FBS and 25, 50, 100 µg /ml of lyophilized *Lagenaria siceraria* juice sample for 24 h. The cytotoxicity assay was performed by measuring the viability of cells using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide)-based method. The viability

was determined in relation to control cells cultured in sample-free media. All experiments were repeated at least three times, and standard error values were <5%.

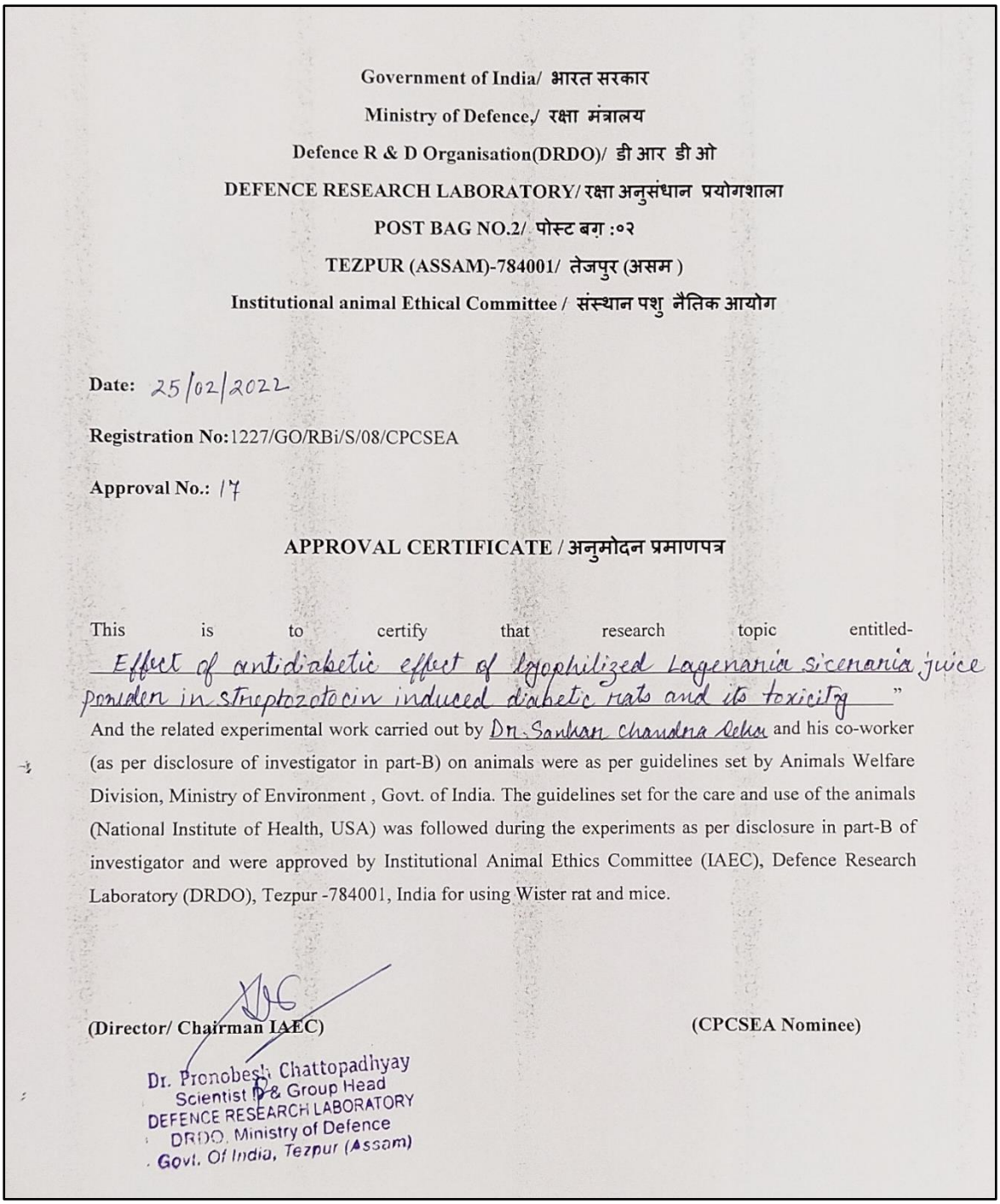
(C) Cell viability assay of lyophilized *Lagenaria siceraria* juice in THP-1 human monocyte cell line

In vitro cytotoxicity of lyophilized *Lagenaria siceraria* juice sample was performed using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide) based method in THP-1 cell line. $2X10^3$Cells were seeded RPMI-1640 supplemented with 10% FBS in 96 well plate [4]. The cells were treated with FBS and 25, 50, 100 µg /ml of lyophilized *Lagenaria siceraria* juice sample for 24 h. Cytotoxicity was determined by adding 10 µl of MTT (5 mg/ml in PBS) to each well and incubated for 3-4 h. The medium was removed and 200 µl DMSO was added to each well and after 10 min of mechanical shaking, the optical density was measured at 570-690 nm in microplate reader (Thermo Scientific Multiskan GO). The viability was determined with respect to the control cells cultured in drug-free media. All experiments were repeated at least three times.

4.2.1.2. *In vivo* acute oral toxicity study of lyophilized *Lagenaria siceraria* juice

4.2.1.2.1. Ethical permission for *in vivo* acute oral toxicity study

All experimental procedures followed the Animal Ethical Committee, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were approved by Institutional animal Ethical Committee of Defence Research Laboratory, Tezpur, Assam India. with a registration number 1227/GO/Rbi/S/08/CPCSEA and protocol number 17/IAEC/DRL/25/2/2022. Acute oral toxicity study was performed according to the Organization of Economic Co-Operation and Development (OECD) guideline 420 for testing of chemicals (**Figure.4.1.)**.



Government of India/ भारत सरकार

Ministry of Defence,/ रक्षा मंत्रालय

Defence R & D Organisation(DRDO)/ डी आर डी ओ

DEFENCE RESEARCH LABORATORY/ रक्षा अनुसंधान प्रयोगशाला

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TEZPUR (ASSAM)-784001/ तेजपुर (असम)

Institutional animal Ethical Committee / संस्थान पशु नैतिक आयोग

Date: 25/02/2022

Registration No:1227/GO/RBi/S/08/CPCSEA

Approval No.: 17

APPROVAL CERTIFICATE / अनुमोदन प्रमाणपत्र

This is to certify that research topic entitled- "Effect of antidiabetic effect of lyophilized Lagenaria siceraria juice powder in streptozotocin induced diabetic rats and its toxicity"

And the related experimental work carried out by Dr. Sankar Chandra Deka and his co-worker (as per disclosure of investigator in part-B) on animals were as per guidelines set by Animals Welfare Division, Ministry of Environment , Govt. of India. The guidelines set for the care and use of the animals (National Institute of Health, USA) was followed during the experiments as per disclosure in part-B of investigator and were approved by Institutional Animal Ethics Committee (IAEC), Defence Research Laboratory (DRDO), Tezpur -784001, India for using Wister rat and mice.

(Director/ Chairman IAEC)

Dr. Pronobesh Chattopadhyay
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(CPCSEA Nominee)

Figure.4.1: Certificate from Animal Ethical Committee, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by Institutional animal Ethical Committee of Defence Research Laboratory, Tezpur, Assam India.

4.2.1.2.2. Experimental design for the *in vivo* acute oral toxicity study

Male Wistar rats (Rattus norvegicus) weighing between 130 and 160 g were used For the acute toxicological experiments. The animal house at the Defence Research Laboratory in Tezpur, Assam, India, was where we were able to get the rats. Before beginning the actual trials, the animals spent seven days becoming used to the environment of the laboratory. The rats were housed in an environment with a constant temperature of 22–24 ºC, a light/dark cycle lasting 12 hours, and a humidity level of about (50± 5%). During the acclimatisation process, the rats were randomly divided into experimental and control groups and kept separately in sterilised polypropylene cages with sterile rice husk as bedding. This was done to ensure that the results of the experiment were as accurate as possible. The animals had unrestricted access to a regular pellet feed as well as water on a continuous basis. Rats between 6 and 8 weeks old were fasted for 16 hours and were utilised. The lyophilized juice of *Lagenaria siceraria* was dissolved in water and given to the rats (n = 3) just once, orally, at a single dosage of 2000 mg/kg at a rate of 20 ml/kg. After that, all of the rats were given unrestricted access to food and water, and they were monitored for a period of 24 hours. During the first 4 hours and once per day over the next 14 days, extra attention was paid to looking for any symptoms of acute toxicity. The visual observations of mortality, different changes in physical appearance, behaviour (salivation, lethargy), and any injuries or illnesses were carried out once per day over a period of 14 days [9](Figure. 4.2.).

Figure. 4.2: Male wistar rats (*Rattus norvegicus*)

4.2.1.2.3. Animal sacrifice for the *in vivo* acute oral toxicity study

On day 15, ketamine was injected intraperitoneally into all of the animals in order to put them under anaesthesia. After performing a heart puncture, blood samples were then collected into tubes containing EDTA and tubes that were not heparinized for further haematological and biochemical examination, respectively. At the Defense Research Laboratory in Tezpur, which is located in the state of Assam in India, haematological and biochemical tests were carried out. After then, ketamine was injected intraperitoneally into the rats in order to put them to sleep. The organs, namely the liver, heart, spleen, lung, and kidney, were removed with with care and then weighed. For the purpose of histological investigation, these organs were maintained in a fixation solution consisting of 10% buffered formalin. The following formula was used to determine each animal's proportionate organ weight:

Relative organ weight = (organ weight (g)/body weight of the animal on sacrifice day (g)) × 100

4.2.1.3. *In vivo* subacute oral toxicity study of lyophilized *Lagenaria siceraria* juice

4.2.1.3.1. Ethical permission for *in vivo* subacute oral toxicity study

All experimental procedures followed the Animal Ethical Committee, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were approved by Institutional animal Ethical Committee of Defence Research Laboratory, Tezpur, Assam India. with a registration number 1227/GO/Rbi/S/08/CPCSEA and protocol number 17/IAEC/DRL/25/2/2022. Subacute oral toxicity study was performed according to the Organization of Economic Co-Operation and Development (OECD) guideline 407 for testing of chemicals and World Health Organization guideline [9].

4.2.1.3.2. Experimental design for the *in vivo* subacute acute oral toxicity study

In the study, researchers employed a total of twelve male rats as subjects. Subacute toxicity study of lyophillized *Lagenaria siceraria* juice at the doses of 250, 500, and 1000 mg/kg body weight were administered orally to three groups, respectively, at every 24 h for 28 days; controls received no dose of lyophillized *Lagenaria siceraria* juice. The results of the acute toxicity study indicated that lyophillized *Lagenaria siceraria* juice was nontoxic

at the dose level of 2000 mg/kg. There were many other harmful indications and observations that were kept an eye on [9], such as body weight, death rate, and the amount of food and drink that was consumed.

4.2.1.3.3. Weekly Body Weight

The body weight of each rat was carefully recorded before the study and once weekly during the study, and on the day of the sacrifice.

4.2.1.3.4. Mortality and Toxic Signs

The visual observations of mortality, various changes in physical appearance, behaviour (sleepy, salivation, lethargy), and any injury or illness were conducted once daily for 28 days, especially after dosing and up to 4 h after dosing [12].

4.2.1.3.5. Animal sacrifice for the *in vivo* acute oral toxicity study

After a period of 28 days, all the animals that were still alive had a fasting period overnight followed by anaesthesia. A cardiac puncture was performed in order to obtain blood samples, which were then placed in either heparinized tubes or non-heparinized tubes for further haematological and biochemical investigation, respectively. After blood was collected from the rats, the animals were sacrificed, and their internal organs (including their hearts, livers, spleens, kidneys, and lungs) were removed, then weighed to determine the relative weights of each organ, and examined for any gross abnormalities. For the purpose of histological investigation, the internal organs were maintained in a 10% buffered formaldehyde solution [5].

4.2.1.3.6. Haematological Parameters

Following the collection of blood from the heart puncture into tubes containing EDTA, many parameters were examined at the Defence Research Laboratory at Tezpur, Assam, India. The haematological parameters included haemoglobin (Hb), red blood cells (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total white blood cells (WBCs), differential white blood cells (neutrophil, lymphocyte, and

monocyte), platelet count, red blood cell distribution unit (RDW), platelet distribution width (PDW)

4.2.1.3.7. Biochemical Estimations

The non-heparinized tubes containing the blood were then subjected to a 10-minute centrifugation at 3000 r/min. At the Defence Research Laboratory in Tezpur, Assam, India, the separated serum was examined for a number of different parameters, including sodium, potassium, chloride, creatinine, urea, uric acid, total protein, albumin, globulin, albumin-globulin ratio, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALPT), and total bilirubin [5].

4.2.1.3.8. Histopathology Study

The organs, namely the liver, heart, spleen, lung, and kidney, were removed with care and weighed after the procedure. For the histological investigation, these organs were maintained in a fixation solution consisting of 10% buffered formalin. Following the procedure outlined in [3], the organ paraffin slices were produced, stained with haematoxylin and eosin, and prepared for examination using a light microscope.

4.2.2. *In vitro* and *in vivo* anti-diabetic study of lyophilized *Lagenaria siceraria* juice

4.2.2.1. *In vitro* α-amylase inhibition study of lyophilized *Lagenaria siceraria* juice

The α -amylase inhibitory activity was evaluated using the techniques described in [18], with a few minor adjustments made in accordance with [8]. Lyophilized *Lagenaria siceraria* juice with known concentrations (50, 100, or 200 g/ml) was combined with 70 µL of enzyme solution (18 units/ml) and the volume was brought up to 1 ml with 0.02 M sodium phosphate buffer (pH 6.9). The reaction mixture was pre-incubated for ten minutes at a temperature of 25 °C. After that, 500 µL of a starch solution with a concentration of 1% was added, and the reaction mixture was left to rest at 25 °C for half an hour. After incubation, 0.5 millilitres of dinitrosalicylic acid reagent were added, which brought an end to the reaction (1g 3, 5-dinitrosalicylic acid in 20 ml of 2 M NaOH and 30 g Rochelle salt in 100 ml distilled water). The test tubes were heated in a boiling water bath for five minutes before being removed, cooled to room temperature, and then diluted five times

with distilled water before having their absorbance measured at 540 nm. The control was made in the same manner as described above, but no extract was used.

4.2.2.2. *In vitro* α-glucosidase inhibition study of lyophilized *Lagenaria siceraria* juice

The lyophilized juice of *Lagenaria siceraria* was tested for its ability to inhibit α -glucosidase using a modified version of the procedure described in [17]. In 96-well microplates, a volume of 50 µl of lyophilized *Lagenaria siceraria* juice at varying concentrations (50, 100, and 200 µg/ml) and 100 l of α -glucosidase (SRL, Mumbai, India) solution (1.0 U/ml) in 0.1 M phosphate buffer (pH 6.9) were mixed together, and the plates were then incubated at 25 °C for 10 minutes. After the first step of preincubation, 50 µl of 5 mM p-nitrophenyl -D-glucopyranoside (Sigma, St. Louis, MO, USA) in 0.1 M phosphate buffer (pH 6.9) was added to each well. The mixture was then incubated at 25 °C for 5 minutes. The plate reader was used to determine the absorbance at a wavelength of 405 nm (BioTek, Mumbai, India).

4.2.2.3. Determination of DPP-4 inhibition activity of lyophilized *Lagenaria siceraria* juice

The DPP-4 inhibition experiment was performed on lyophilized *Lagenaria siceraria* juice of several concentrations (20, 50, 100, and 200) on a black 96-well plate according to the methodology provided by the manufacturer and the procedure published by [7,16]. Dipeptidyl-peptidase 4, also known as CD26 and adenosine deaminase complexing protein-2, is a membrane glycoprotein that has serine exopeptidase activity. It is responsible for cleaving X-proline dipeptides off the N-terminus of polypeptide chains. Oral anti-diabetic medicines that are inhibitors of DPP4 have evolved in recent years. These medications prevent the breakdown of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 that is caused by DPP-4. The DPP-4 Inhibitor Screening Kit is an easy assay that is well-suited for high-throughput screening of compounds that have the potential to inhibit DPP4. Cleaving the substrate in order to produce a luminous product (lex = 360/ lem = 460 nm), which is proportional to the amount of enzymatic activity that is present, is how the DPP4 activity is assessed.

4.2.2.4. *In vitro* Glucose uptake assay on L6 rat skeletal muscle cell line

It was from the National Centre for Cell Science in Pune, India, that we were able to acquire the L6 skeletal muscle cell line. Cells were grown in DMEM supplemented with 10% FBS and incubated at 370 ºC in a humidified environment containing 5% carbon dioxide. The DMEM included 100 units of penicillin and 100 mg of streptomycin per ml of medium. L6 myoblasts underwent differentiation into L6 myotubes two days after confluence had occurred. In a nutshell, L6 myoblasts were grown in DMEM that had been supplemented with 10% FBS, 5% CO2, 370 degrees Fahrenheit, 10,000 units per ml of penicillin, 10 mg per ml of streptomycin, and 25µg ml/1 of amphotericin B. The chamber was humidified. After two to three passages of L6 myoblasts, the cells were then allowed to develop and fuse into myotubes in a culture medium containing two percent foetal bovine serum (FBS). The differentiation of myotubes was seen under a microscope, and all of the studies were carried out on cells that had differentiated to their utmost potential (more than 85%) seven days after confluence.

The glucose uptake test was carried out with the assistance of a glucose uptake cell-based assay kit (Cayman, USA), in accordance with the instructions provided by the manufacturer. In a nutshell, L6 myotubes were stored in DMEM without glucose for a full 24 hours. Following this, the cells were pretreated with lyophilized *Lagenaria siceraria* juice of varying concentrations for one hour before being subjected to an incubation with palmitate (0.75 mM) for four hours. Thirty minutes before to the conclusion of the incubations, the cells were treated with insulin (100 nM). Before stopping the experiment, a fluorescent label glucose analogue 2-NBDG was placed and applied to each of the incubations for a period of ten minutes. After that, the cells were lysed, Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland) was used to measure the fluorescence intensity [2].

4.2.2.5. *In vivo* anti-diabetic study of lyophilized *Lagenaria siceraria* juice

4.2.2.5.1. Induction of diabetes and experimental design

After an overnight fast of eight hours, the animals in each group were given an intraperitoneal injection of a single dosage of streptozotocin (STZ) equal to 55 mg/kg BW dissolved in 0.2 ml of freshly made 0.1M citrate buffer at pH 4.5 [9, 10]. This caused the

animals to develop diabetes. The development of diabetes was verified by a fasting blood glucose (FBG) test three days after the STZ solution was used to induce it. The test involved the tail pricking of blood droplets and the use of a handheld glucometer. Rats whose FBG levels were more than 6 mmol/L were considered eligible for the research and were enrolled in it.

The rats that made up group I (the control) were healthy animals who did not have diabetes. Diabetic rats were used for the study of group II, which received no therapy. Insulin was administered to the rats in group IV, while rats in group III were given a dose of lyophilized *Lagenaria siceraria* juice that was newly re-constituted in deionized water at a rate of 600 mg/kg of body weight (Figure. 4.3.). The dosage of the lyophilized *Lagenaria siceraria* juice was selected in line with the results of the toxicity studies.

Figure. 4.3: Male wistar rats (*Rattus norvegicus*) for anti-diabetic study.

4.2.2.5.2. Animal weights

4.2.2.5.3. Fasting blood glucose

After being subjected to the therapy for a period of four weeks, the rats were fasted overnight prior to the experiment, after which they were given glucose at a rate of 2.5 g/ kg. The tail vein was used to assess the levels of glucose in the blood (Figure. 4.4.).

Figure. 4.4: The blood glucose levels checked from the tail vein of Male wistar rats (*Rattus norvegicus*)

4.2.2.5.4. Oral glucose tolerance test (OGTT)

After being subjected to the therapy for a period of four weeks, the rats were fasted overnight prior to the experiment, after which they were given glucose at a rate of 2.5 g/kg. After the glucose challenge, the tail vein was used to measure the blood glucose levels and compare them to those measured earlier (0, 30, 60, 90, 120 min).

4.2.2.5.5. Intraperitoneal insulin tolerance test (IPITT)

Following a treatment period of 4 weeks, rats were allowed to fast for a period of 6 h before having insulin injected intraperitoneally. The tail vein was used to measure the blood glucose levels at 0 minutes (just before the insulin was given), 30, 60, 90, and 120 minutes after the insulin was given.

4.2.2.5.6. Animal sacrifice

On day 31 of treatment, the animals were humanely euthanized by an overdose of halothane (5% by volume in oxygen), and blood was collected by cardiac puncture in lithium heparinized tubes. The blood was then separated into plasma and stored at a temperature of -80 degrees Celsius prior to being analysed. For the purpose of studying gene expression, gastrocnemius muscles were removed, washed in normal saline, frozen

in liquid nitrogen, and then kept at a temperature of -80 degrees Celsius. A phosphate-buffered formalin solution was used to preserve the liver and pancreas for histological examination after they were removed surgically and cut into equal halves. The halves were then snap-frozen in liquid nitrogen.

4.2.2.5.7. Histopathology of Pancreas

In preparation for histological examination, the pancreas that had been removed was stored in a fixation solution consisting of 10% buffered formalin. Following the procedure outlined in [3], the organ paraffin slices were produced, stained with haematoxylin and eosin, and prepared for examination using a light microscope.

4.2.3. Anti-inflammatory study of lyophilized *Lagenaria siceraria* juice

4.2.3.1. In vitro LPS induced inflammation study in THP-1 macrophage

THP-1 monocytes were obtained from the National Centre for Cell Science in Pune, India. They were then cultured in RPMI1640 medium containing penicillin (100 U/ml) and streptomycin (100 mg/ml) and supplemented with 10% foetal bovine serum at a temperature of 37°C in an atmosphere containing 5% carbon dioxide. Samples of lyophilized *Lagenaria siceraria* juice were tested for their ability to inhibit inflammation caused by LPS in THP-1 macrophages. Real Time PCR analysis of TNF-α and IL-1 β (mRNA level in THP-1 macrophage pre-treated with or without *Lagenaria siceraria* juice in varied concentrations were checked, in presence or absence of LPS (100ng/ml) for 4 hours [2].

4.2.3.2. Luciferase reporter assay

THP-1 macrophages, at a density of 1 x 10^4 cells/ well, were transfected with B promoter luciferase plasmid at a concentration of 0.1 µg/ well using Lipofectamine 3000 Transfection Reagent in accordance with the procedure provided by the manufacturer. In a nutshell, 0.3 µl of Lipofectamine 3000 Transfection Reagent and 0.1 µg of plasmid DNA were added to 10 µl of Opti-MEM medium. The two solutions were combined, and the mixture was then incubated for 10 minutes. The transfection mixture was applied to the cells when they were in the presence of RPMI 1640 medium devoid of antibiotics. Following a six-hour incubation at 37 °C, the culture medium was changed to RPMI 1640,

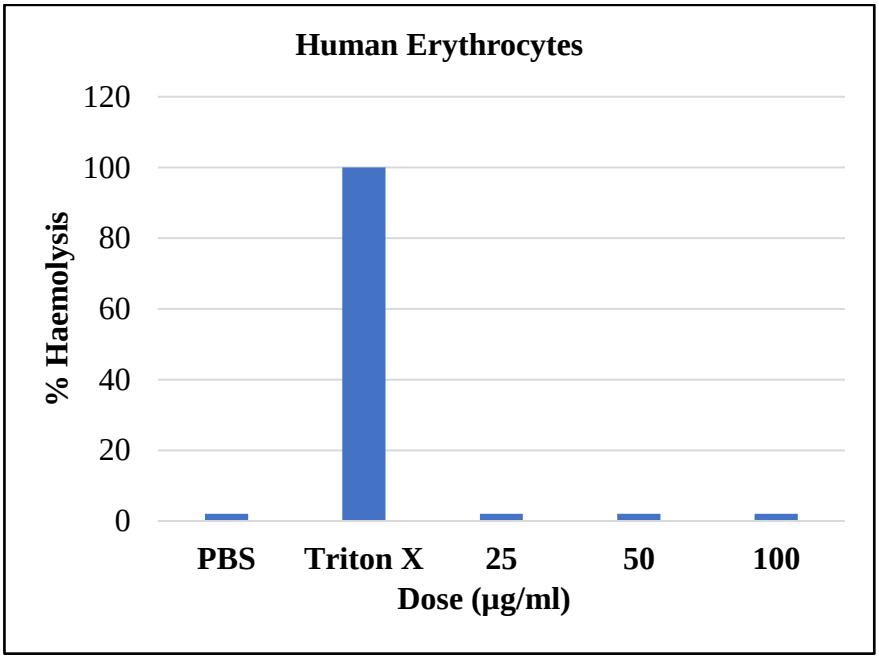
which included 20% FBS foetal bovine serum (FBS). After a transfection time of 48 hours, the cells were washed with RPMI 1640 and then put through incubations. After the completion of the incubations, the THP-1 macrophages were lysed, and the luciferase activity was determined using a Steady-Glo Luciferase Assay System (Thermo Scientific, Finland) in conjunction with a Varioskan LUX Multimode Microplate Reader [2].

4.3. Results

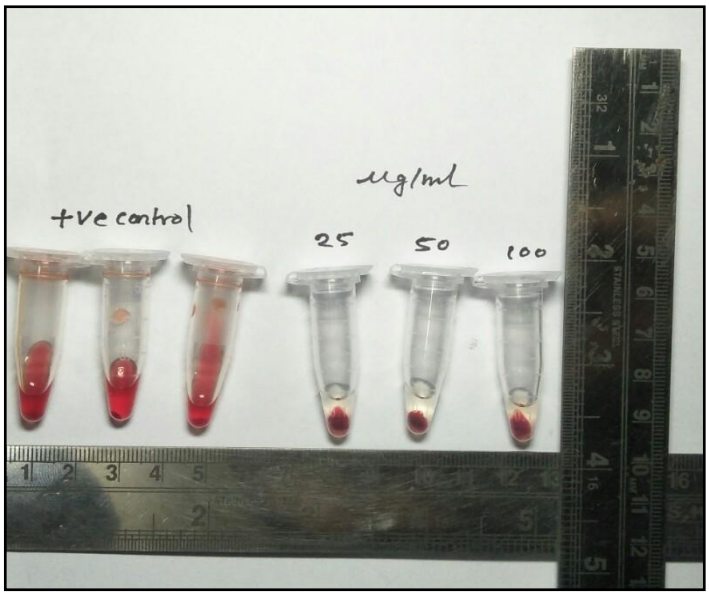
4.3.1. Toxicity study of lyophilized *Lagenaria siceraria* juice

(A) Cell viability assay of lyophilized *Lagenaria siceraria* juice in Human Erythrocytes

The effects of different concentration of lyophilized *Lagenaria siceraria* juice on % inhibition of haemolysis of erythrocytes incubated in hypotonic solution was observed. No haemolysis was observed in RBC at a tested dose of 25 µg, 50 µg and 100 µg per ml of solution for bottle gourd extract (**Figure. 4.5.)**. The result shows that lyophilized *Lagenaria siceraria* juice is not toxic to human erythrocytes.



(a)



(b)

Figure. 4.5: (a) Effect of different concentration of lyophilized *Lagenaria siceraria juice* on % inhibition of haemolysis of erythrocytes incubated in hypotonic solution. (b) Picture representing haemolysis after incubated with lyophilized *Lagenaria siceraria* juice (0-100 µg/ml) w.r.t. positive control (Triton X-100) (n=3). Reading at 535 nm

(B) Cell viability assay of lyophilized *Lagenaria siceraria* juice in Human peripheral blood mononuclear cells (HPBMC)

The effects of different concentration of lyophilized *Lagenaria siceraria* juice on MTT based cell viability assay in HPBMC was observed. Human PBMC were viable at a tested dose 5 µg, 10 µg, 25 µg, 50 µg and 100 µg per ml of solution for lyophilized *Lagenaria siceraria* juice incubated for 24 h (**Figure. 4.6.)**. The result shows that lyophilized *Lagenaria siceraria* juice is not toxic to human peripheral blood mononuclear cells.

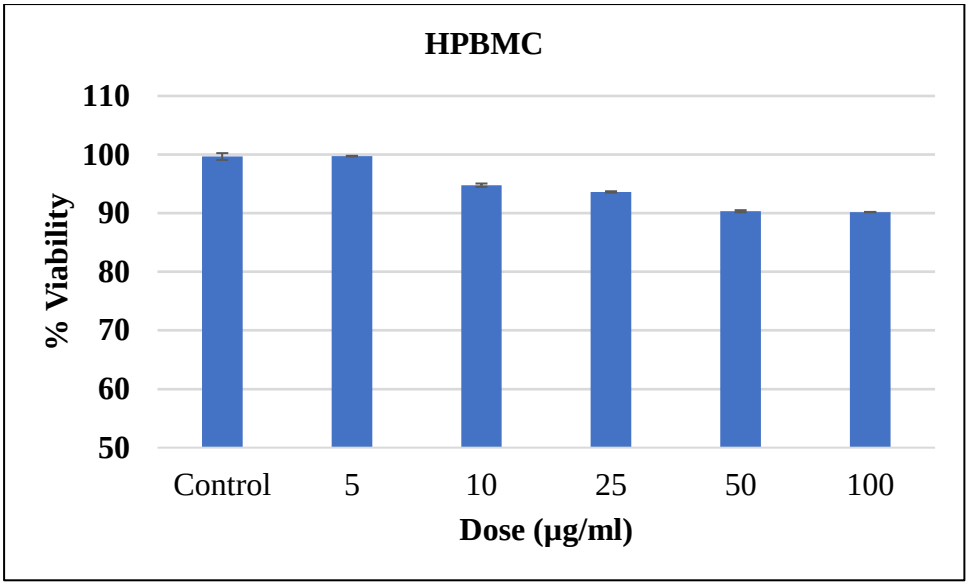


Figure. 4.6: Results of cell viability assay of (a) LS (*Lagenaria siceraria*) in HPBMCs in 0-100 µg/ml for 24 h measured by MTT based method. The viability is calculated as % of control and finally expressed as Mean±SD (n=3).

Histopaq buffer was used for separation of PBMCs. Human PBMC were viable at a tested dose 5 µg, 10 µg, 25 µg, 50 µg and 100 µg per ml of solution for lyophilized *Lagenaria siceraria* juice incubated for 24 h.

(C) Cell viability assay of lyophilized *Lagenaria siceraria* juice in THP-1 human monocyte cell line

The effects of different concentration of lyophilized *Lagenaria siceraria* juice on MTT based cell viability assay in in THP-1 human monocyte cell line was observed. Human monocytes were viable at a tested dose 5 µg, 10 µg, 25 µg, 50 µg and 100 µg per ml of solution for lyophilized *Lagenaria siceraria* juice incubated for 24 h (**Figure. 4.7.)**. The result shows that lyophilized *Lagenaria siceraria* juice is not toxic to human peripheral blood mononuclear cells.

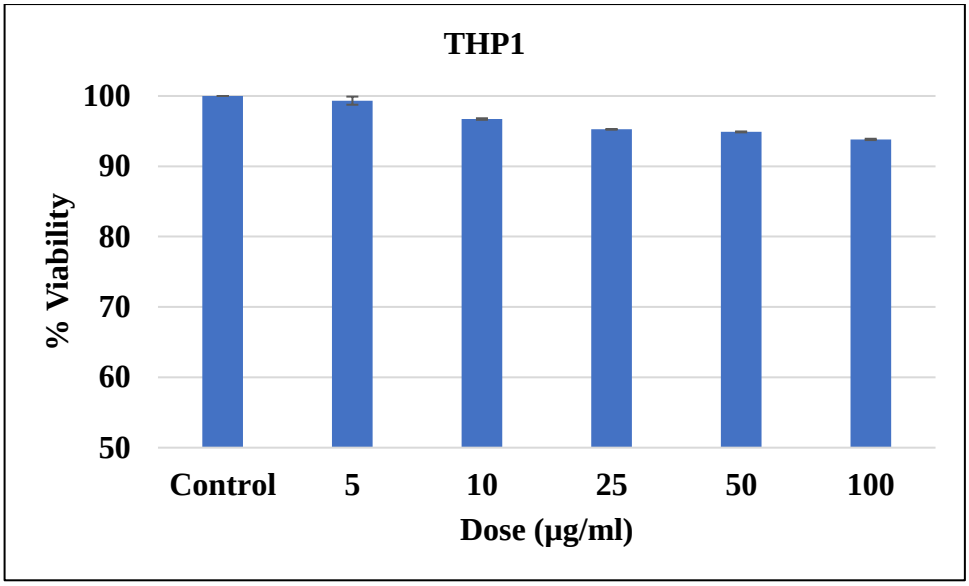


Figure. 4.7: Results of cell viability assay of lyophilized *Lagenaria siceraria* juice in THP-1 in 0-100 µg/ml for 24 h measured by MTT based method. The viability is calculated as % of control and finally expressed as Mean±SD (n=3).

4.3.2. Effect of lyophilized *Lagenaria siceraria* juice on Wistar rats in acute oral toxicity study.

Lyophillized *Lagenaria siceraria* juice at a dose of 2000 mg/kg produced no toxic effect on the behavioural responses of the treated rats (dosed once) and observed for 14 days. There were no signs of changes in the behaviour patterns, skin, eyes, salivation, and diarrhoea of the rats. Neither mortality nor significant weight loss was observed. There were generally no significant differences observed in the relative organ weight (**Table. 4.1.)**. From the present study it was seen that there was no significant change in the haematological (**Table. 4.2.)** and biochemical parameters (**Table. 4.3.)** in the *Lagenaria siceraria juice* treated group compared to control group. The histopathological (**Figure. 4.8, 4.9)** evaluations of various organs stained with haematoxylin and eosin revealed no significant differences. Although some differences has been observed, the haematological and biochemical parameters showed no significant differences in the physiological parameters.

Effect of the lyophilized *Lagenaria siceraria* juice on haematological parameters, biochemical parameters and histopathology was conducted of the vital organs of the

animal models in both acute and subacute oral toxicity study. No signs of toxicity were noticed in the experimental animal models.

Table. 4.1: The relative organ weight of rats treated with a single dose of lyophilized *Lagenaria siceraria* juice for 14 days in acute oral toxicity study

SL No	Organs	Control	LS (2000mg/kg)
1	Liver	4.64±05	4.40±04
2	Kidney	0.92±25	0.99±24
3	Brain	1.00±04	1.10±06
4	Spleen	0.34±02	0.40±09
5	Heart	0.45±04	0.48±02
6	Lungs	0.79±28	0.72±32
7	Stomach	1.52±42	1.06±25

Values are expressed as the mean ± SD (standard deviation) (n = 3; for each group);. Relative organ weight was calculated as (organ weight (g)/body weight of animal on sacrifice day (g)) × 100.

Table. 4.2: Effect of lyophilized *Lagenaria siceraria* juice on haematological parameters in acute oral toxicity study

SL No	Parameter	Unit	Control	Lyophilized *Lagenaria siceraria* juice (2000mg/kg)
1	Total white blood cells (WBC's)	10^9 /L	11.10 ±0.02	10.96±0.15
2	Lymphocytes	10^9 /L	6.0±0.01	6.36±0.25
3	Monocyte	10^9 /L	0.4±0.01	0.41±0.01
4	Granulocyte	10^9 /L	5.2±0.01	5.36±0.15
5	Lymphocyte %	%	51.5±0.49	51.0±0.20
6	Monocyte %	%	3.2±0.01	3.2±0.1
7	Granulocyte %	%	45.18±0.20	46.8±0.1
8	Total red blood cells (RBC's)	10^{12}/L	8.49±0.05	8.44±0.04
9	Haemoglobin	g/L	147.13±0.32	147±0.2
10	HCT	%	43.83±0.35	43.2±0.2
11	Mean Corpuscular	IL	51.23±0.30	51.3±0.2

	volume (MCV)			
12	Mean Corpuscular haemoglobin (MCH)	pg	17.53±0.15	17.4±0.2
13	Mean Corpuscular haemoglobin concentration (MCHC)	g/L	340.1±0.36	340.16±0.47
14	RDW	%	14.73±0.32	14.5±0.1
15	Platelet count	10^9 /L	567.76±0.25	586±0.5
16	Mean latelet volume	IL	5.83±0.15	5.8±0.2
17	Platelet distribution width	%	16.2±0.2	16.1±0.2
18	Procalcitonin	%	0.324±0.005	0.339±0.006

Values are expressed as the mean ± SD (n = 3; for each group).

Table. 4.3: Effect of lyophilized *Lagenaria siceraria* juice on biochemical parameters in acute oral toxicity study

SL No	**Parameter**	**Unit**	**Control**	**Lyophilized *Lagenaria siceraria* juice (2000mg/kg)**
1	Urea	mg/dL	31.3±0.2	31.20±0.1
2	Creatinine	mg/dL	0.30±0.01	0.29±0.007
3	Total Protein	g/dL	5.67±0.15	5.70±0.11
4	Albumin	g/dL	2.90±0.2	2.86±0.15
5	A/G Ratio		0.90±0.01	1.00±0.05
6	AST	U/L	95.00±0.15	94.00±0.28
7	ALTV	U/L	58.00±0.15	60.00±0.26
8	ALKP	U/L	122.00±0.25	124.00±0.58
9	Total Bilirubin	mg/dL	0.20±0.01	0.19±0.01
10	Globulin	g/dL	3.0±0.15	2.8±0.1

Values are expressed as the mean ± SD (n = 3; for each group).

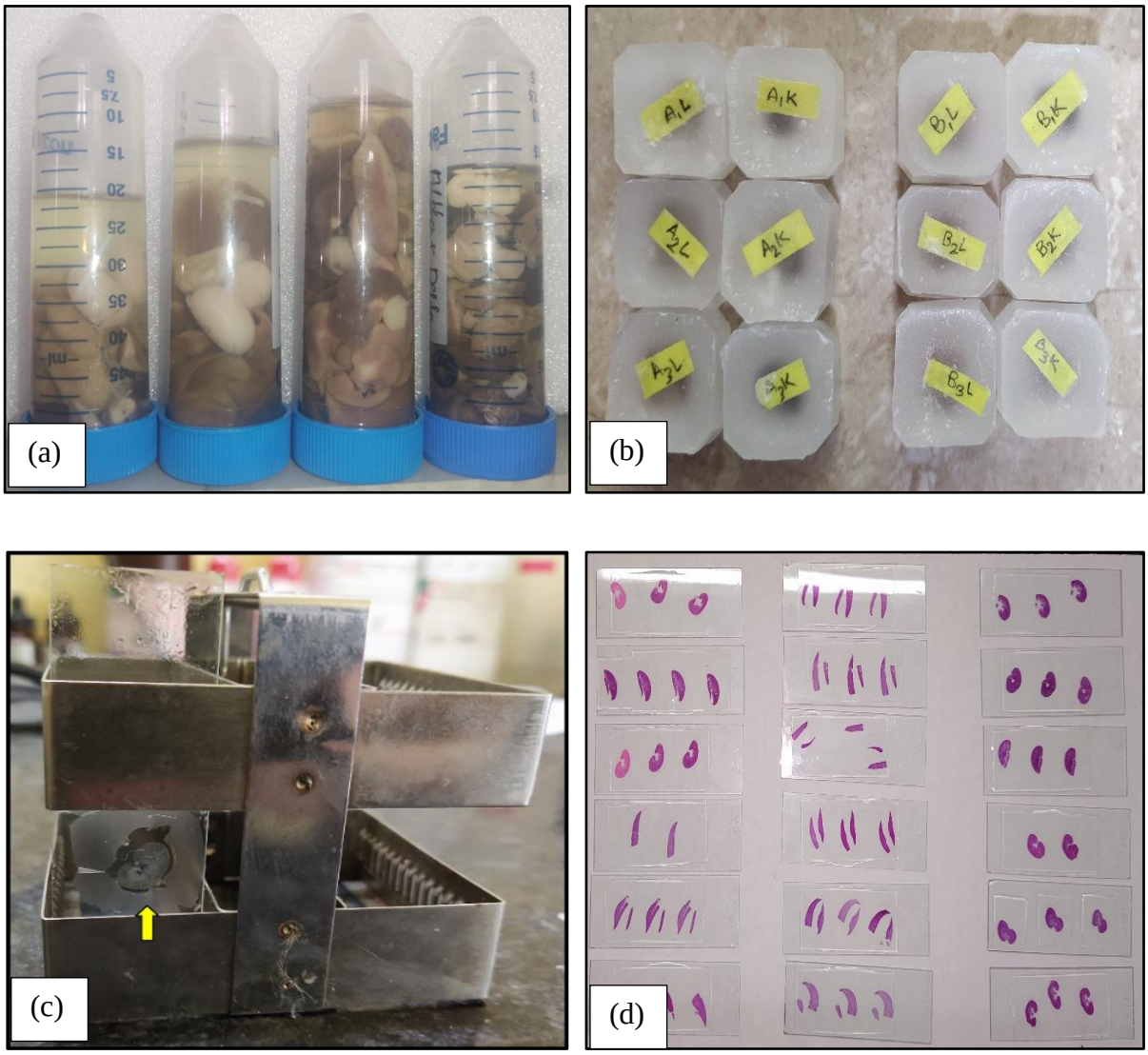


Figure. 4.8: (a) Wistar rats' organs preserved in a fixation medium of 10% buffered formalin for histopathological study.

(b) The organ paraffin blocks.

(c) The microtome cut organ paraffin section (arrow showing section of kidney).

(d) The organ sections stained with haematoxylin and eosin, and processed for light microscope

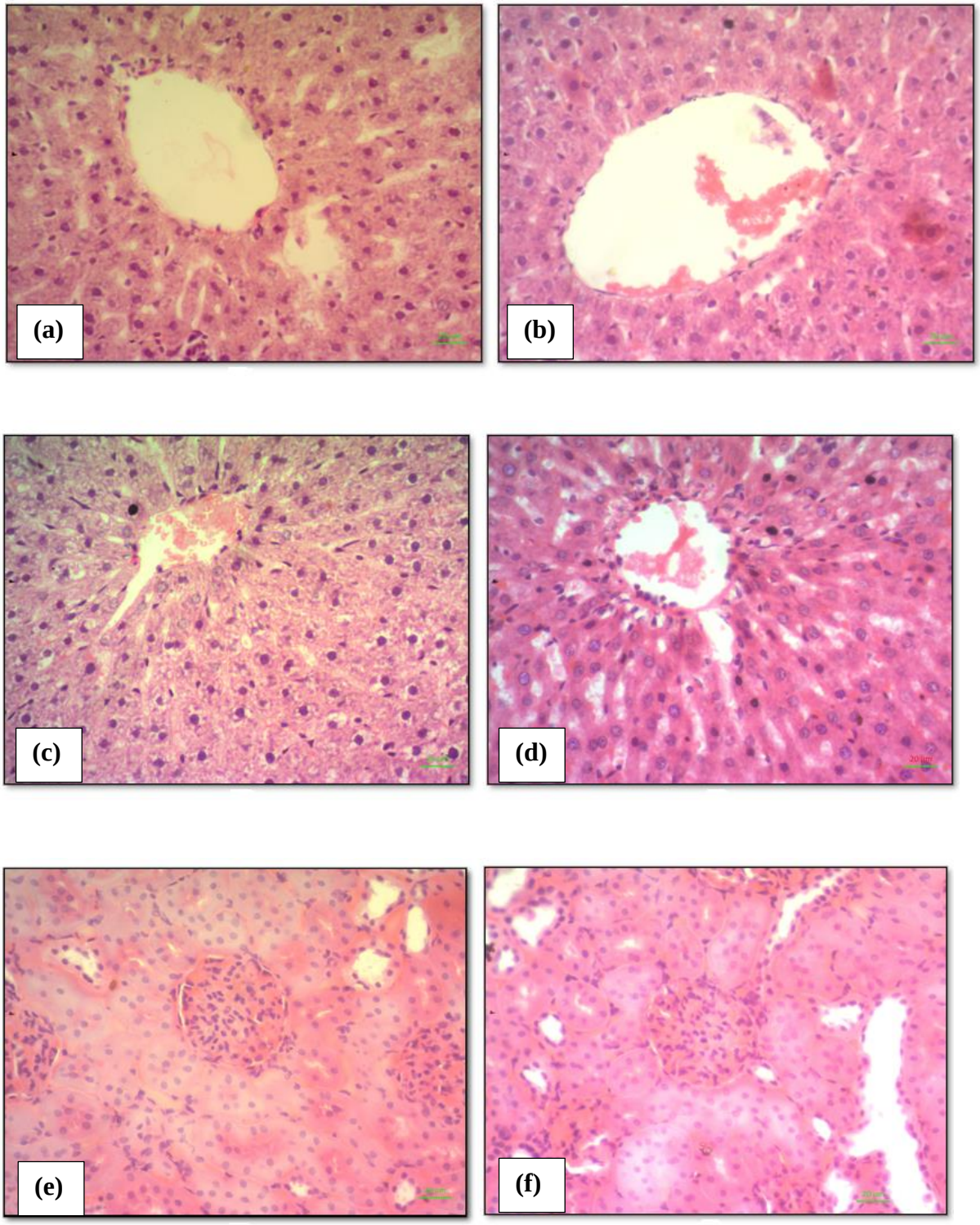


Figure. 4.9: Histopathological examination of Liver and Kidney of control and treated rats of the acute oral toxicity study

(a). Control group liver section showing central vein
(b). Liver section of lyophilized *Lagenaria siceraria* juice treated group showing central vein
(c). Control group liver section showing portal vein
(d). Liver section of lyophilized *Lagenaria siceraria* juice treated group showing portal vein
(e). Control group kidney section
(f). Lyophilized *Lagenaria siceraria* juice treated group kidney section

4.3.3. Effect of lyophilized *Lagenaria siceraria* juice on Wistar rats in sub-acute oral toxicity study.

Lyophillized *Lagenaria siceraria* juice at a dose of 250, 500, 1000 mg/kg per day (28 days) produced no toxic effect on the behavioural responses of the treated rats and was observed for 28 days. There were no signs of changes in the behaviour patterns, skin, eyes, salivation, and diarrhoea of the rats. Neither mortality nor significant weight loss was observed (**Table. 4.4.)**. There were generally no significant differences observed in the relative organ weight (**Table. 4.5.)**. From the present study it was seen that there was no significant change in the haematological (**Table. 4.6.)** and biochemical parameters (**Table. 4.7.)** in the *Lagenaria siceraria juice* treated group compared to control group. The kidney function parameters, like urea, creatinine, and uric acid, did not reveal any significant changes. No statistically significant differences in the liver function parameters like alanine aminotranferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were observed. Additionally, no relevant changes were found in total protein, albumin, and globulin.

The histopathological evaluations of various organs stained with haematoxylin and eosin revealed no significant differences (**Figure. 4.10.)**. Although some differences has been observed, the haematological and biochemical parameters showed no significant differences in the physiological parameters. Effect of the lyophilized *Lagenaria siceraria* juice on haematological parameters, biochemical parameters and histopathology was conducted of the vital organs of the animal models in both acute and subacute oral toxicity study. No signs of toxicity were noticed in the experimental animal models.

Table. 4.4: The effect of lyophilized *Lagenaria siceraria* juice on body weight of rats (g) at different days during the sub-acute oral toxicity study.

Group	**Doses**	**Weight (g)**			
		Day 1	Day 9	Day 18	Day 28
	Control	171.±0.5	177±0.25	182±0.25	186±0.17
1	250mg/kg	154.±0.15	158±0.15	161±0.25	164±0.1
2	500mg/kg	188±0.15	194±0.2	199±0.1	202±0.56
3	1000mg/kg	163±0.20	168±0.15	172±0.3	176±0.30

LS (*Lagenaria siceraria*)

Group I-III: The lyophilized *Lagenaria siceraria* juice with different doses;

A weekly body weight was determined on initial (0), 9^{th},18^{th}, and 28^{th} days of four groups.

No significant changes in the body weight were observed.

Table. 4.5: The relative organ weight of rats treated with different doses of the lyophilized *Lagenaria siceraria* juice for 28 days of sub-acute oral toxicity study.

SL No	Organs	Control	250mg/kg	500mg/kg	1000mg/kg
1	Liver	3.79±0.02	3.01±0.02	3.22±0.02	4.30±0.13
2	Kidney	0.82±0.01	0.86±0.005	0.82±0.01	0.89±0.01
3	Brain	0.90±0.01	0.86±0.01	0.87±0.01	0.90±0.01
4	Spleen	0.20±0.007	0.21±0.01	0.23±0.01	0.30±0.1
5	Heart	0.41±0.01	0.37±0.76	0.33±0.01	0.42±0.01
6	Lungs	0.50±0.01	0.63±0.01	0.59±0.01	0.62±0.01
7	Stomach	0.77±0.01	0.89±0.01	0.79±0.01	0.96±0.01

Relative organ weights of 28-day treated rats are shown in Table. The relative organ weight of each organ recorded at necropsy in the treatment groups did not show a significant difference ($p > 0.05$) compared to control.

Table. 4.6: Effect of the lyophilized *Lagenaria siceraria* juice on haematological parameters in sub-acute oral toxicity study.

SL No	**Parameter**	**Unit**	**Control**	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
1	Total white blood cells (WBC's)	10^9 /L	8.6 ±0.12	8.7±0.07	8.8±0.07	8.5±0.15
2	Lymphocytes	10^9 /L	6.2±0.09	5.9±0.05	5.7±0.08	6.0±0.11
3	Monocyte	10^9 /L	0.3±0.01	0.3±0.01	0.3±0.008	0.3±0.02
4	Granulocyte	10^9 /L	3.0±0.06	3.1±0.09	3.2±0.065	2.8±0.05
5	Lymphocyte %	%	68.6±0.15	65.6±0.15	66.3±0.13	65.9±0.14
6	Monocyte %	%	3.2±0.01	4.3±0.03	2.8±0.20	2.9±0.11
7	Granulocyte %	%	33.0±0.01	35.1±0.07	35.9±0.02	31.2±0.17
8	Total red blood cells (RBC's)	10^{12}/L	7.68±0.03	6.49±0.16	6.81±0.57	6.73±0.07
9	Haemoglobin	g/L	128±0.25	121±0.5	122±0.40	129±0.57
10	HCT	%	39.7±0.25	34.1±0.07	37,7±0.15	36.5±0.24
11	Mean Corpuscular volume (MCV)	IL	52.8±0.25	52.6±0.25	51.1±0.15	54.3±0.19
12	Mean Corpuscular haemoglobin (MCH)	pg	17.9±0.18	18.6±0.19	17.9±0.15	19.1±0.11
13	Mean Corpuscular haemoglobin	g/L	347±0.57	354±0.50	351±.0.57	353±0.57

	concentration (MCHC)					
14	RDW	%	12.5±0.2	14.3±0.13	13.5±0.110	12.0±0.13
15	Platelet count	10^9 /L	1074±0.57	950±1.15	1026±0.5	982±1.15
16	Mean latelet volume	IL	5.1±0.1	5.7±0.07	5.0±0.16	5.7±0.07
17	Platelet distribution width	%	15.6±0.1	16.0±0.25	15.5±0.24	15.9±0.20
18	Procalcitonin	%	0.547±0.01	0.513±0.01	0.563±0.006	0.559±0.03

Table. 4.7: Effect of the lyophilized *Lagenaria siceraria* juice on biochemical parameters in subacute oral toxicity study

SL No	**Parameter**	**Unit**	**Control**	***L. siceraria* juice (250mg/kg)**	***L. siceraria* juice (500mg/kg)**	***L. siceraria* juice (1000mg/kg)**
1	Urea	mg/dL	34.5±0.1	37.30±0.15	32.20±0.13	35.20±0.36
2	Creatinine	mg/dL	0.40±0.01	0.39±0.005	0.41±0.01	0.38±0.02
3	Total Protein	g/dL	6.10±0.1	5.90±0.12	5.98±0.08	6.00±0.15
4	Albumin	g/dL	2.90±0.05	3.00±0.10	3.10±0.07	3.00±0.09
5	A/G Ratio		1.05±0.07	1.10±0.12	1.20±0.11	1.00±0.01
6	AST	U/L	109.00±0.51	108.00±0.41	101.00±0.56	109.00±0.35
7	ALTV	U/L	63.00±0.35	64.00±0.36	62.00±0.45	62.00±0.24
8	ALKP	U/L	136.00±0.50	129.00±0.56	134.00±0.45	135.00±0.35
9	Total Bilirubin	mg/dL	0.20±0.01	0.20±0.008	0.19±0.01	0.18±0.5
10	Globulin	g/dL	3.10±0.15	3.20±0.04	3.30±0.04	3.0±0.06

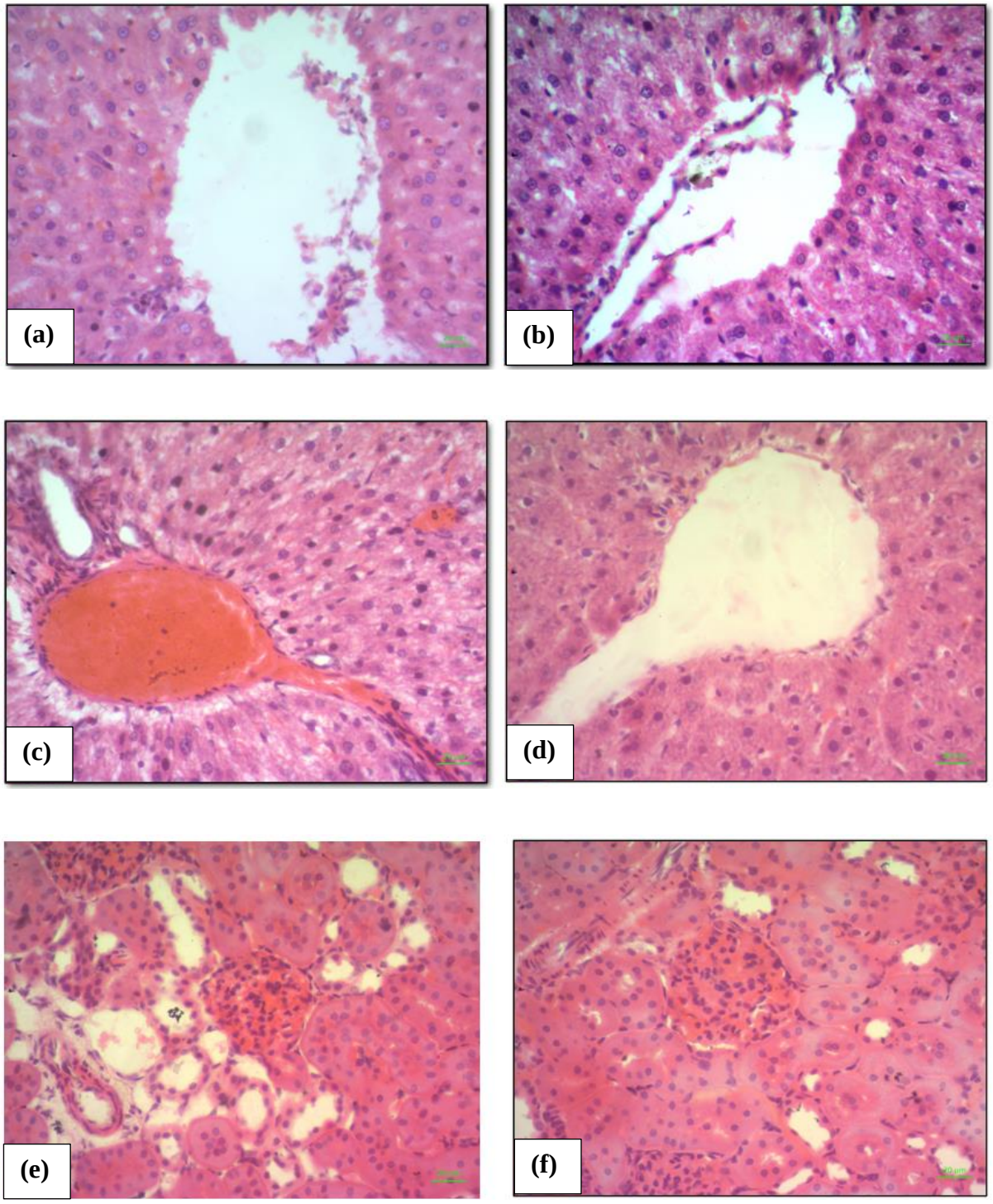


Figure. 4.10: Histopathological examination of Liver and Kidney of control and lyophilized *Lagenaria siceraria* juice treated rats (1000mg/kg) of the sub-acute oral toxicity study

(a). Control group liver section showing central vein

(b). Liver section of lyophilized *Lagenaria siceraria* juice treated group showing central vein

(c). Control group liver section showing portal vein

(d). Liver section of lyophilized *Lagenaria siceraria* juice treated group showing portal vein

(e). Control group kidney section

4.3.4. Inhibition of α-amylase enzyme activity

The health beneficial effects of *Lagenaria siceraria* juice was investigated for its antidiabetic potential using *in vitro* enzyme inhibitory assays. The *in vitro* α-amylase inhibition study revealed that *Lagenaria siceraria* juice showed significant α-amylase inhibition at all the tested concentrations (**Figure. 4.11.)**. 200µg of lyophilized *Lagenaria siceraria* juice showed the highest inhibition of over 22% of α amylase enzyme.

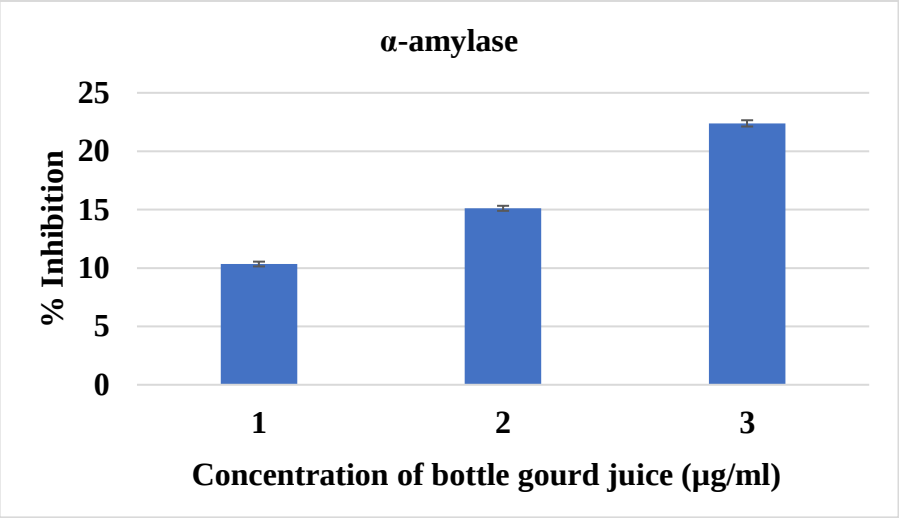


Figure. 4.11: Inhibition of α-amylase enzyme activity

4.3.5. Inhibition of α-Glucosidase enzyme activity

The *in vitro* α-glucosidase inhibition study revealed that *Lagenaria siceraria* juice showed significant α-glucosidase inhibition at all the tested concentrations. 200µg of lyophilized *Lagenaria siceraria* juice showed the highest inhibition of over 50% of α-Glucosidase enzyme (**Figure. 4.12.)**. The results supported the beneficial effects of *Lagenaria siceraria* juice for its antidiabetic potential.

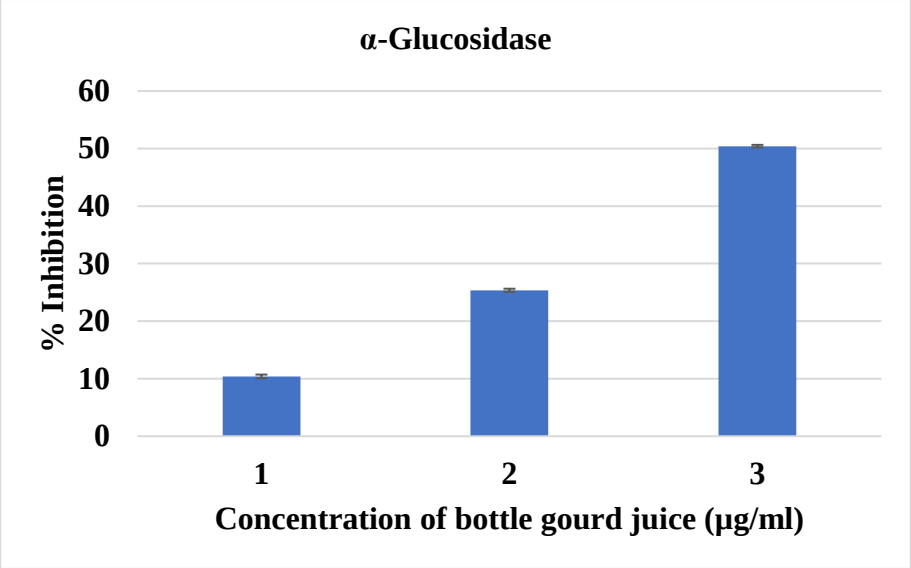


Figure. 4.12: Inhibition of α-Glucosidase enzyme activity

4.3.6. Determination of DPP-4 inhibition activity

The *in vitro* DPP-4 inhibition assay revealed that *Lagenaria siceraria* juice showed significant DPP-4 enzyme inhibition at all the tested concentrations. 200µg of lyophilized *Lagenaria siceraria* juice showed inhibition of DPP4 enzyme activity over 60% (**Figure. 4.13.)**. The results supported the beneficial effects of *Lagenaria siceraria* juice for its antidiabetic potential.

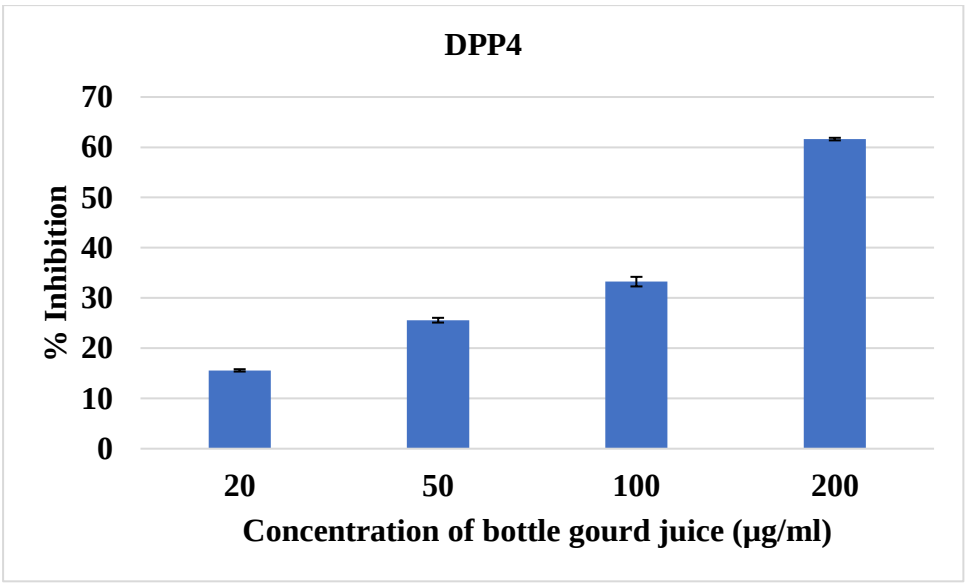


Figure. 4.13: Inhibition of DPP4 enzyme by different concentration of *Lagenaria siceraria* juice

4.3.7. *In vitro* Glucose uptake assay

Insulin mediated 2NBDG uptake was checked using lyophilized *Lagenaria siceraria* juice on L6 rat skeletal muscle cell line at different concentration. 10µg/ml concentration of *Lagenaria siceraria* juice were found to be effective on 2NBDG uptake (**Figure. 4.14.)**.

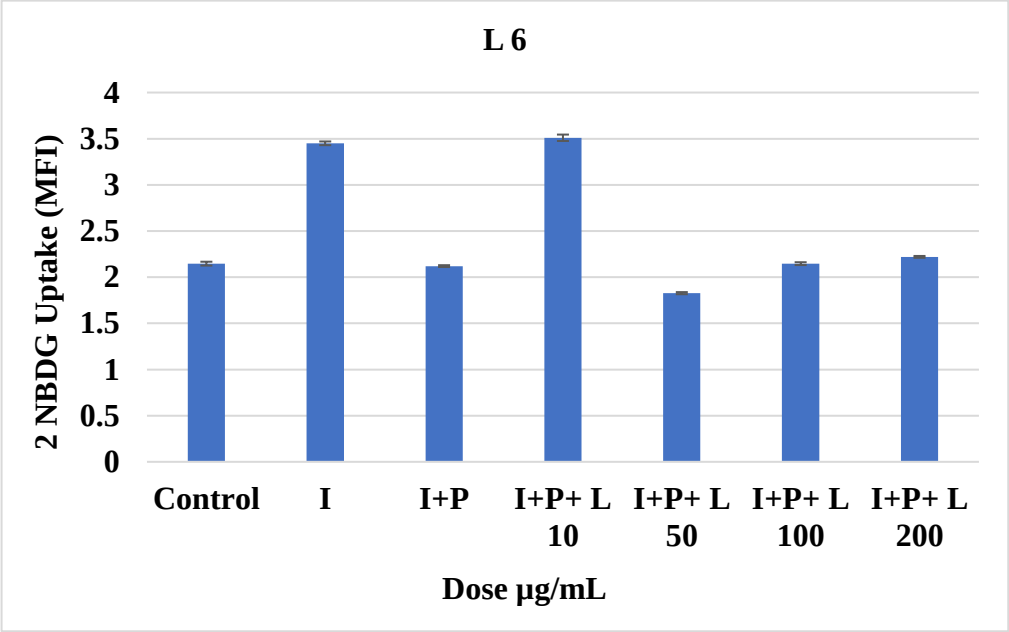


Figure. 4.14: Insulin mediated 2NBDG uptake was enhanced in LS (*Lagenaria siceraria*) juice treated (L6) cells at 10µg/ml concentration'

2NBDG =D-Glucose, 2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl) amino)-

I=Insulin, P=Palmitate, L= Lyophillized *Lagenaria siceraria* juice

4.3.8. *In vivo* diabetic study

4.3.8.1. Animal weights

The control nondiabetic rats had a normal weight gain in the study period. The diabetic animals showed weight after the induction of diabetes. The groups treated with insulin had a slight improvement in normal weight gain. The lyophilized *Lagenaria siceraria* juice treated rats had weight loss after the induction of diabetes but the weight loss was lower than untreated rats (**Figure. 4.15.)**. This shows that *Lagenaria siceraria* juice might have helped the rats to improve their normal metabolism.

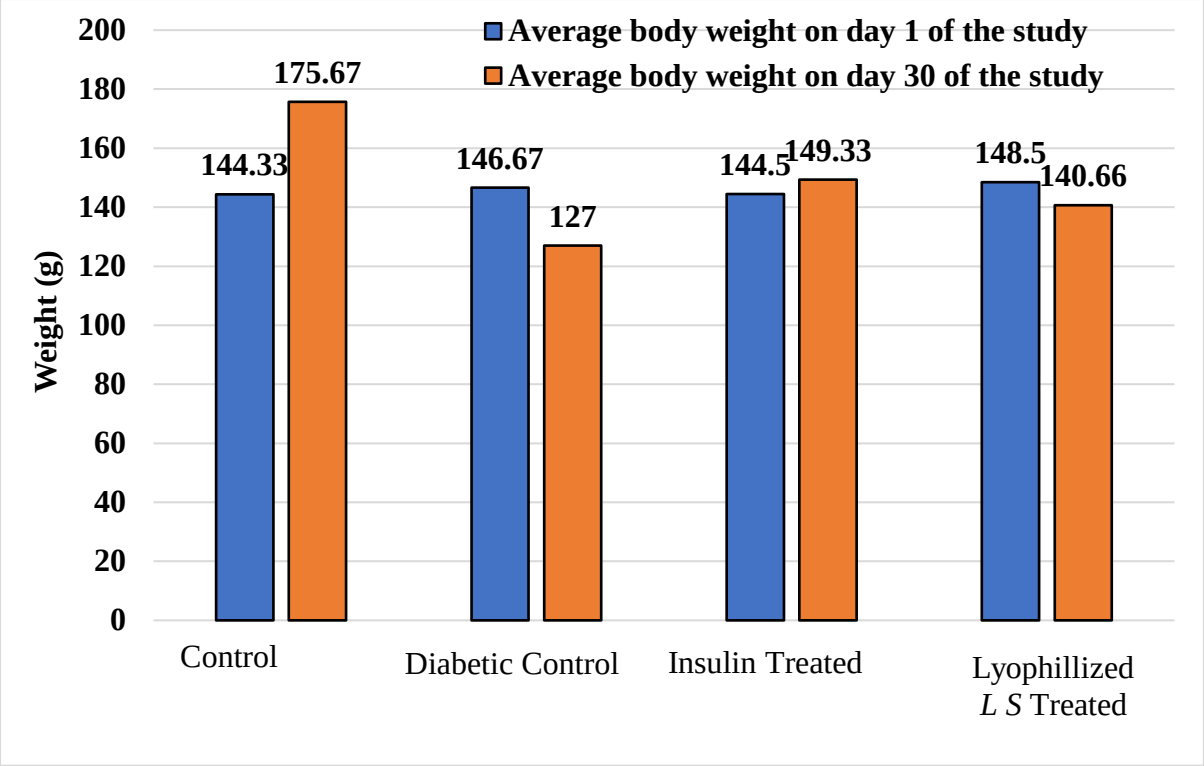


Figure. 4.15: Animal weight change during the anti-diabetic study

LS= *Lagenaria siceraria*

4.3.8.2. Fasting blood glucose

Fasting blood glucose of non-treated diabetic rats was significantly elevated compared to controls. Treatment of diabetic rats with lyophilized *Lagenaria siceraria* juice or insulin significantly lowered fasting blood glucose compared to non-treated diabetic rats (**Figure. 4.16.)**.

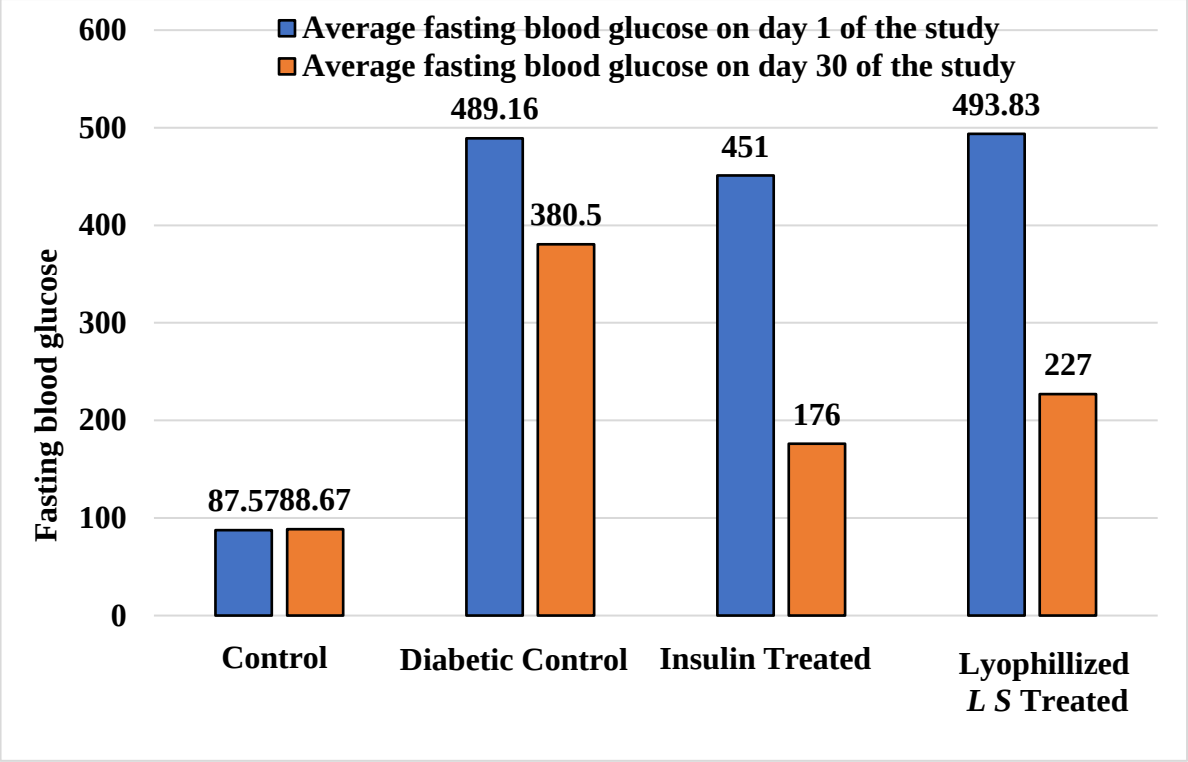


Figure. 4.16: Fasting blood glucose

4.3.8.3. Oral Glucose tolerance test

After the glucose was injected intraperitoneally into all of the groups, there was an immediate and significant rise in the blood glucose level. After two hours, normal control animals and rats fed with lyophilized *Lagenaria siceraria* juice had lower blood glucose concentrations than diabetic animals, and these normal rats returned to their baseline levels. When compared to controls, diabetic rats exhibited a considerable increase in their glucose intolerance (Figure. 4.17.). In comparison to the diabetic group that was not given any treatment, glucose intolerance was dramatically improved when diabetic rats were given either lyophilized juice from *Lagenaria siceraria* or insulin at a dose of 2.0 IU/kg BW.

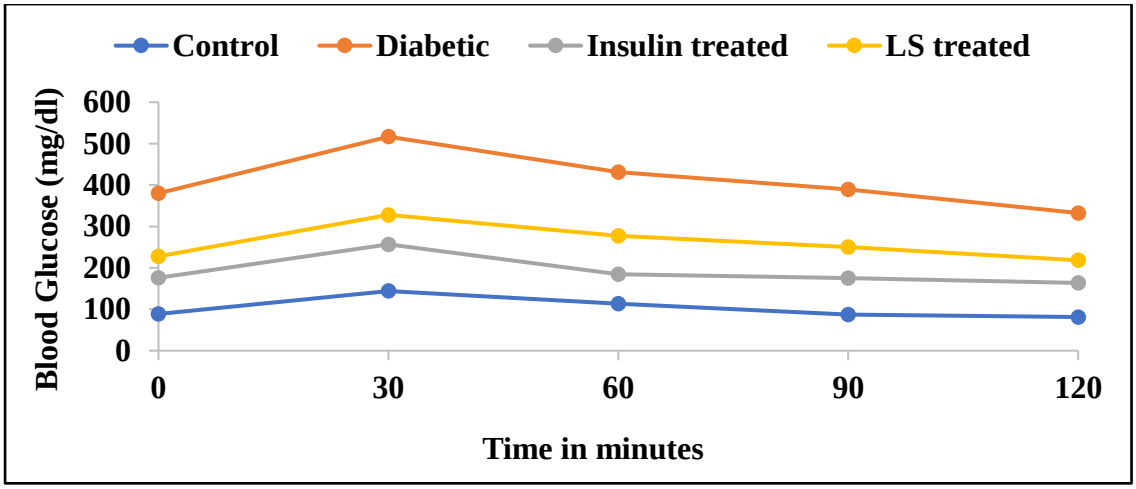


Figure. 4.17: Oral Glucose Tolerance Test

4.3.8.4. Intraperitoneal Insulin Tolerance Test

To evaluate the effect of lyophilized *Lagenaria siceraria* juice on glucose metabolism in wistar rats, intraperitoneal insulin tolerance test (IPITT) was performed. Lyophilized *Lagenaria siceraria* juice treated rats showed a significant decrease of blood glucose levels compared with the non-treated group in response to insulin (**Figure. 4.18.**). Thus, lyophilized *Lagenaria siceraria* could improve insulin tolerance in wistar rats.

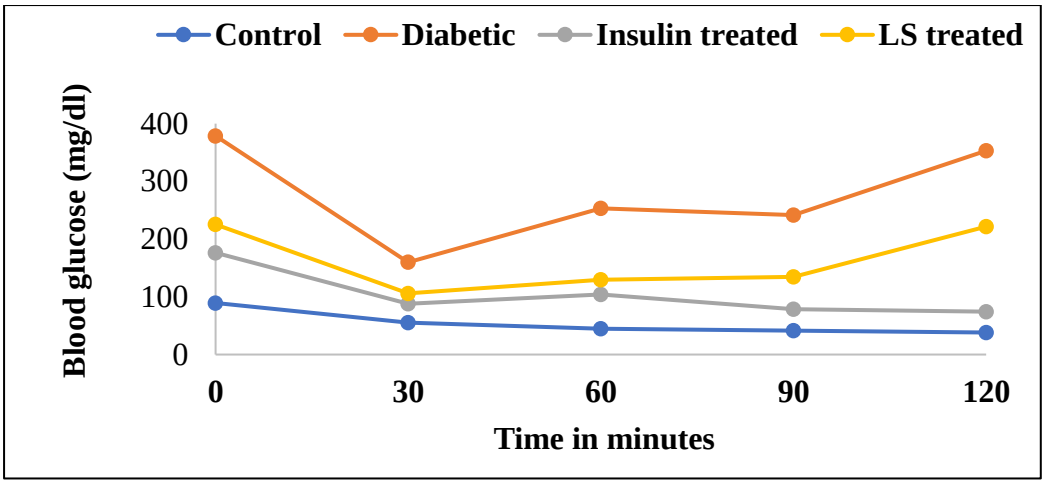


Figure. 4.18: Intraperitoneal Insulin Tolerance Test

4.3.8.5. Animal sacrifice and histopathology of Pancreas

The microscopic investigation of the pancreas showed that the control group displayed normal histomorphology with normal islets of Langerhans cells. The pancreas section of diabetic controls can be seen with abnormal islets of Langerhans cells. The group treated with insulin and the group treated with lyophilliized *Lagenaria siceraria* juice had abnormality in the islets of Langerhans cells with presence of vacuoles. It can be observed that in the two treated groups the condition of the pancreatic islets of Langerhans cells are much better than the diabetic control group. This shows the effectiveness of *Lagenaria siceraria* juice in repairing the condition of the pancreatic cells **(Figure. 4.19.).**

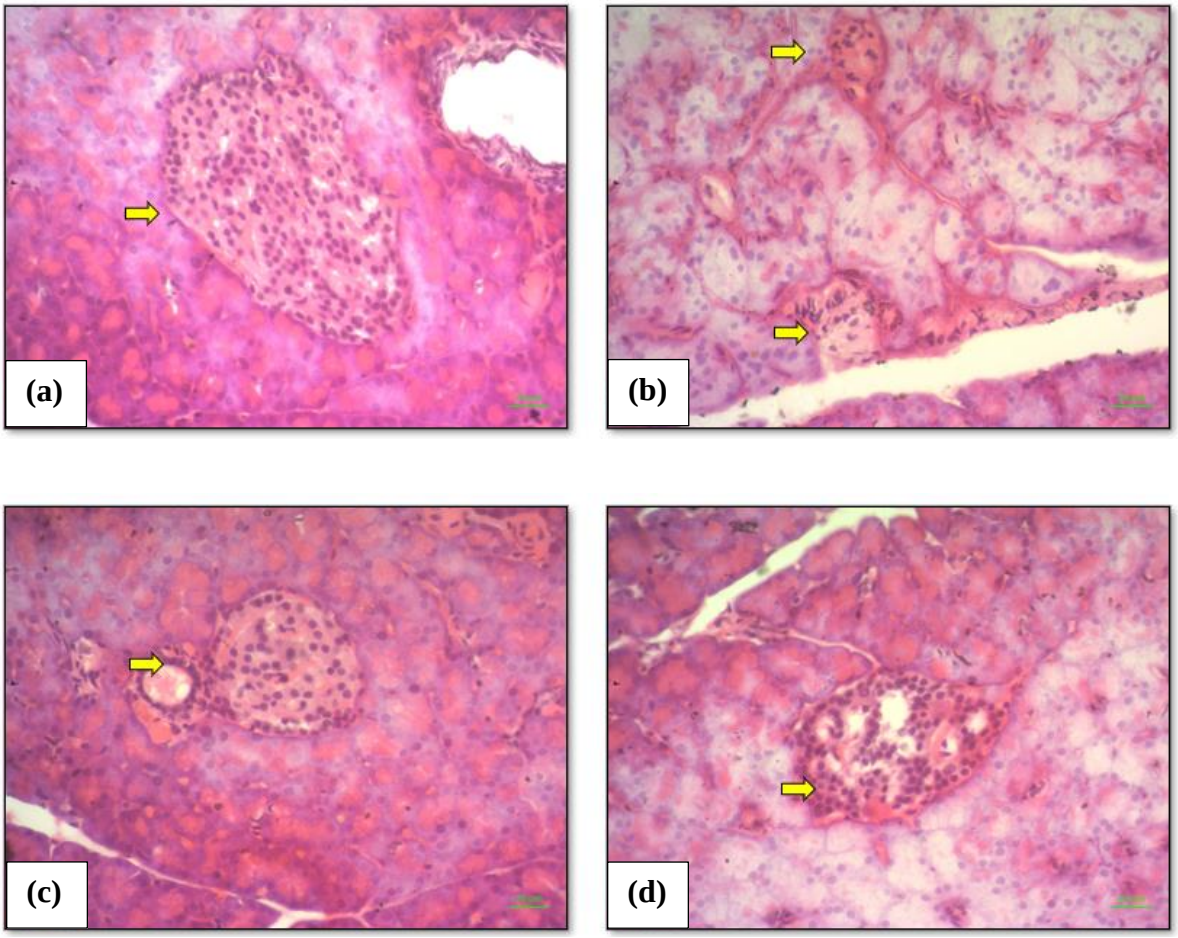


Figure. 4.19: Histopathology of pancreas

(a). Control group displaying normal histomorphology of the pancreas (arrow showing normal islets of Langerhans cells.

(b). Diabetic control group (arrow showing abnormality in the islets of Langerhans cells).

(c). Group treated with insulin (arrow showing abnormality in the islets of Langerhans cells with presence of vacuoles).

(d). Group treated with lyophilized *Lagenaria siceraria* juice (arrow showing abnormality in the islets of Langerhans cells with presence of vacuoles).

4.3.9. In vitro LPS induced inflammation study in THP-1 macrophage

The THP-1 cells pre-treated with *Lagenaria siceraria* juice showed notable reduction in TNFa and IL-1b gene expression in presence of LPS, where LPS strongly upregulated TNFa and IL-1b mRNA level in absence of *Lagenaria siceraria* juice **(Figure. 4.20, 4.21.).** The luciferase activity assay also revealed that NF-kB gene expression of THP-1 cells in presence of LPS were reduced in presence of *Lagenaria siceraria* juice. These result supports that *Lagenaria siceraria* juice have potential anti-inflammatory property **(Figure. 4.22.).**

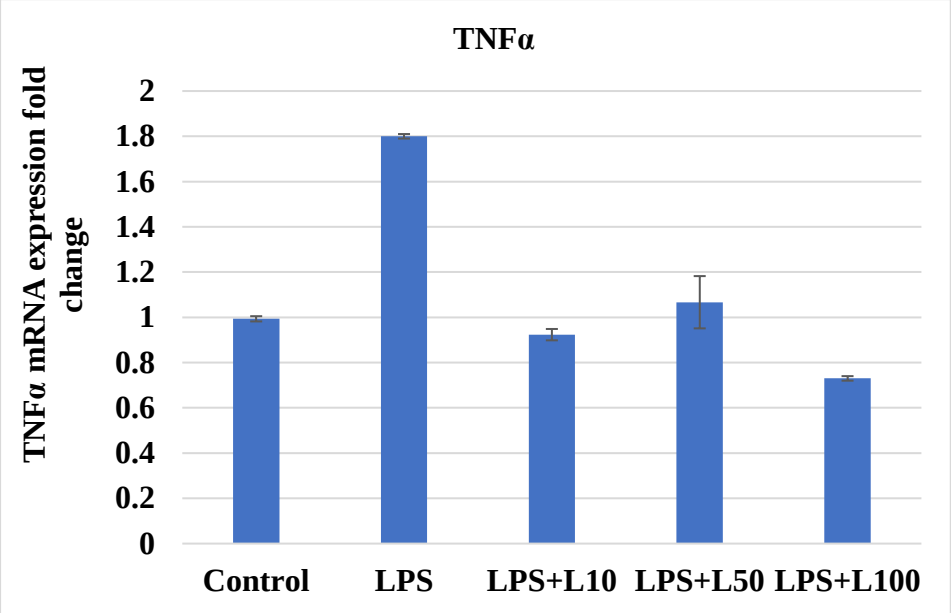


Figure. 4.20: Real Time PCR analysis showing TNFα mRNA level in THP-1 macrophage pre-treated with or without *Lagenaria siceraria* juice in varied concentration, in presence or absence of LPS (100ng/ml) for 4 h.

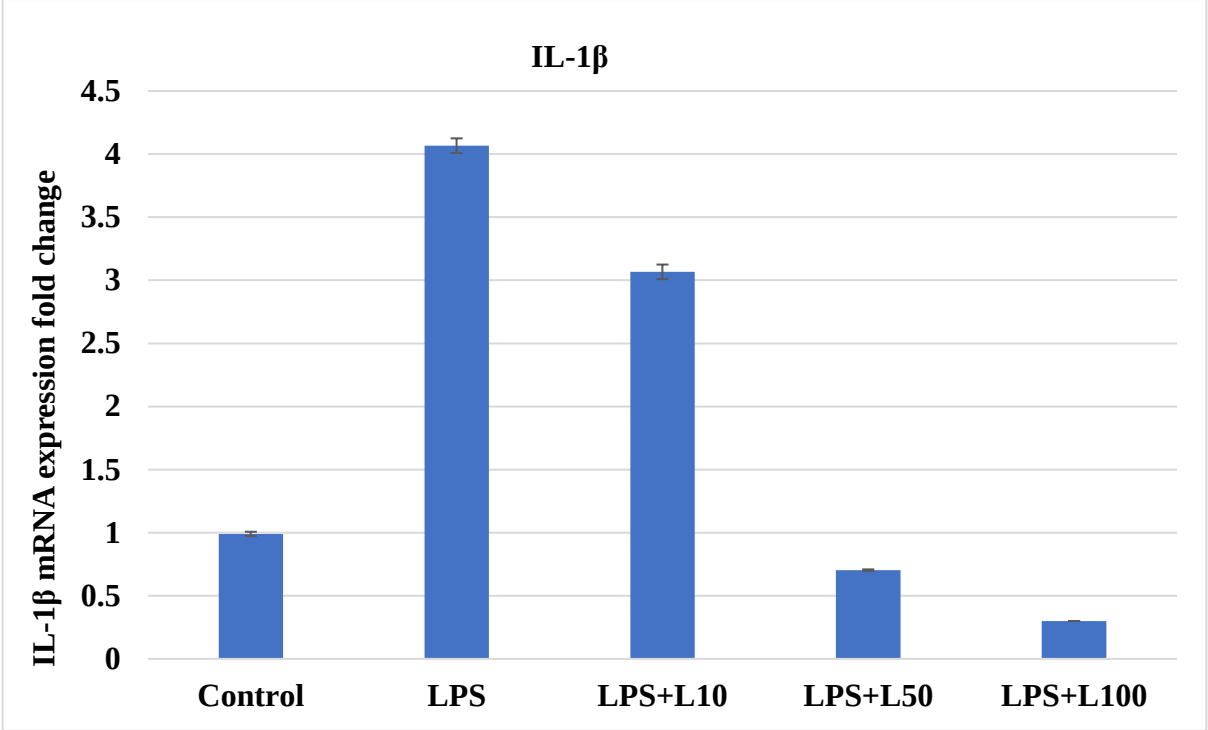


Figure. 4.21: Real Time PCR analysis showing IL-1β mRNA level in THP-1 macrophage pre-treated with or without *Lagenaria siceraria* juice in varied concentration, in presence or absence of LPS (100ng/ml) for 4 h.

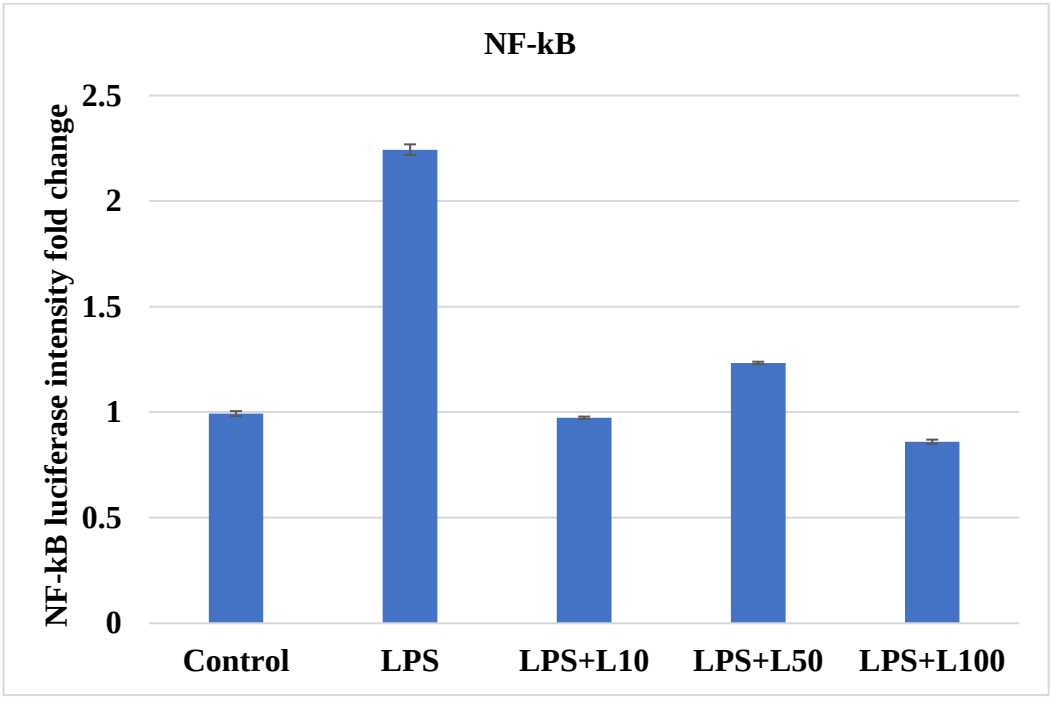


Figure. 4.22: Luciferase activity measured for NF-kB gene on LPS induced inflammation in THP-1 macrophage with or without *Lagenaria siceraria* juice in varied concentration

4.4. Conclusions

The study revealed that *Lagenaria siceraria* juice is nontoxic to human erythrocyte and HPBMCs, THP-1 human monocyte cell line. After the *in vivo* toxicity study, it was also revealed that the *Lagenaria siceraria* juice is nontoxic to wistar rats. The *in vitro* and *in vivo* antidiabetic study revealed the health beneficial potentiality of *Lagenaria siceraria* juice. *Lagenaria siceraria* juice was also found effective in the anti-inflammatory study.

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