CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Development, Safety Evaluation, and Antidiabetic Activity of Propolis-*Nigella sativa* Nanoemulsion

by

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A thesis submitted in partial fulfillment for the degree of Master of Science

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In pursuit of knowledge, progress, and meaningful discovery.

CERTIFICATE OF APPROVAL

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Abstract

The rising burden of diabetes mellitus worldwide necessitates the development of effective and affordable therapeutic strategies. This study formulated and characterized a lipid nanoemulsion incorporating propolis and *Nigella sativa* extracts, using oleic acid, Tween 80, and soy lecithin as lipid and surfactant components. The nanoemulsion exhibited an optimal particle size, a pH of 4.8, a zeta potential of -23, and good thermal and visual stability over 21 days.

The emulsion's safety profile was assessed in *Sprague dawley* rats over 14 days, followed by an antidiabetic evaluation in a diabetic rat model. Toxicological analysis revealed no significant impact on alanine aminotransferase (ALT), bilirubin levels, or body weight at lower doses. However, a mild increase in alkaline phosphatase (ALP) was observed at higher doses, indicating the need for further safety assessment. In the antidiabetic evaluation, nanoemulsion-treated diabetic rats demonstrated improved renal function parameters and superior glycemic control compared to untreated diabetic controls. The enhanced therapeutic effect is attributed to the bioactivity of propolis and *Nigella sativa*, known for their antioxidant, anti-inflammatory, and antidiabetic properties.

These findings suggest that the nanoemulsion displayed limited toxicity at low doses and exerted a significant antidiabetic effect in the diabetic rat model. The aim of this study was to synthesize, characterize, and evaluate the safety profile and antidiabetic activity of a lipid nanoemulsion incorporating propolis and *Nigella sativa* extracts.

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Abbreviations

ALP	Alkaline phosphatase
ALT	Alaline transaminase
DPP-4	Dipeptidyl Peptidase-4 inhibitors
FTIR	Fourier Transform Infrared Spectroscopy
GLUT	Glucose Transporter
HbA1c	Hemoglobin A1c (Glycated Hemoglobin)
HLB	Hydrophobic lipophilic balance
IL-1	Interleukin-1
IL-7	Interleukin-7
SGLT2	Sodium-Glucose Cotransporter-2
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
TNF	Tumor Necrosis Factor
TZD	Thiazolidinediones

Chapter 1

Introduction

Diabetes mellitus represents one of the most significant global health challenges of our time, affecting billions worldwide. The disease is characterized by persistently high blood glucose levels and can present in two forms, each with distinct underlying causes. Type 1 diabetes is classified as an autoimmune condition, in which auto-antibodies attack and destroy beta cells of the pancreas, leading to insulin deficiency. Lack of insulin triggers the body to begin breaking down fats for energy, leading to the production of ketones, and ultimately, if left untreated, a condition called diabetic ketoacidosis. Symptoms develop rapidly, due to the body's inability to use glucose efficiently and include polyuria, polyphagia, polydipsia, weight loss, fatigue and blurred vision. This form typically presents in early childhood but can develop at any age. Type 2 diabetes accounting for 90-95% of all cases globally and is characterized by insulin resistance. The condition is strongly associated with lifestyle factors and obesity, which trigger the release of proinflammatory cytokines and adipokines from adipose tissue, which interfere with insulin signaling pathways in peripheral tissues, leading to hyperglycemia and microvascular and macrovascular complications [1].

The current treatment of diabetes comprises synthetic medication and lifestyle changes such as diet modification and exercise. Medications that are provided to patients stimulate insulin secretion from beta cells of pancreas and decrease insulin sensitivity. Different classes of drugs used for the management of diabetes

include biguanides, sulphonylureases, DPP-4 inhibitors, SGLT2 inhibitors and insulin therapy. These drugs, while effective have a range of side effects such as gastrointestinal disturbances, lactic acidosis, weight gain, hypoglycemia and renal impairment. Furthermore, they require complicated treatment regimens with lifelong compliance [2].

These limitations have led to interest in plant-based therapeutics or phytotherapy. Herbal medicine has been used for centuries as a part of traditional medicine and has gained attention due to its potential to offer safer, more cost effective and sustainable alternatives to synthetic drugs. Phytochemicals are bioactive compounds produced in plants as a byproduct of secondary metabolism and have been validated scientifically to possess a range of beneficial and pharmacological effects, including antioxidant, antibacterial, antifungal, anti-inflammatory, anticancer, antiviral and cardioprotective properties, and have been used to treat a variety of diseases including diabetes [3, 4].

Propolis and *Nigella sativa* are two such natural products which have been shown to offer benefits in the management of diabetes. Propolis is a waxy resinous substance produced as a by-product of honey and consists of waxes, beeswax and honeybee secretions. It reinforces the hive structure and seals cracks. To harvest propolis beekeepers usually scrape it off from the hives. propolis consists of a wide range of phytochemicals including phenolic compounds, terpenes, ketones, aldehydes and fatty acids and its phytochemical composition can vary greatly depending upon the type of honeybee that produced it, the climate in which it was produced and season in which it was harvested [5]. It has shown to have antioxidant, antidiabetic, anti-inflammatory, cardioprotective, neuroprotective and antimicrobial properties [6].

Nigella sativa, also known as black seed or black cumin has considerable pharmacological activity that makes it valuable in both traditional as well as modern medicine. It is an essential part of Ayurveda, Chinese and Unani medicine and has been used treat a broad spectrum of conditions ranging from respiratory ailments, digestive disorders, skin diseases and cardiovascular issues. One of its notable

benefits is the ability to improve glycemic control by enhancing pancreatic beta cell function and increasing insulin sensitivity [7].

While both propolis and *Nigella sativa* have demonstrated individual therapeutic potential, traditional medicine systems often emphasize the value of combining multiple medicinal plants. This practice is based on the principle of synergism, in which the combined effect is greater than the sum of individual effects [8]. This approach allows targeting of multiple biological pathways simultaneously and has been used in both herbal and synthetic drug regimens [9].

Lipid based nanotechnology offers a new approach to enhance the therapeutic potential of drugs, and overcome issues with solubility, bioavailability and absorption. It includes nanoemulsions, solid lipid nanoparticles, liposomes, nanostructured lipid carriers and lipid polymer hybrid nanoparticles [10]. Lipid nanoemulsions are kinetically stable oil-in-water or water-in-oil emulsions that consist of an oil phase and an aqueous phase which has been stabilized by surfactant or emulsifier. These emulsions range in size from 20-500 nm and have the ability to encase and protect hydrophobic and lipophilic drugs and improve their absorption and bioavailability with the body. These emulsions can be prepared by a combination of low energy and high energy methods, and require careful selection of lipid, surfactant and stabilizer, each of which plays a role in the final properties of the emulsion [11].

This research focuses on three aspects. The first is synthesis and characterization of a nanoemulsion containing combined extracts of propolis and *Nigella sativa*. Unlike most studies on nanoemulsions which focus on a single active ingredient, this study uniquely combines two extracts, offering a new approach to understanding their interaction and stability within a lipid-based system. Characterization can be achieved through evaluation of particle size, FTIR, pH, dilution test, and thermal stability studies.

Secondly, toxicity testing assesses the safety profile of the nanoemulsion, a critical step often neglected in lipid based nano drug research despite its importance in drug development. Finally, the study evaluates the antidiabetic potential of

the formulation to understand its therapeutic efficacy. Both aspects are investigated through histological examination of major organs and analysis of biochemical markers, including liver and kidney function tests, providing valuable information regarding both safety and efficacy of the nanoemulsion.

1.1 Hypothesis

The nanoemulsion containing combined extracts of propolis and *Nigella sativa* may exhibit antidiabetic potential in diabetic rat model, whilst displaying limited toxicity.

1.2 Problem Statement

Diabetes mellitus remains a significant global health challenge, with current synthetic treatments often causing adverse effects and compliance issues. While propolis and *Nigella sativa* show promising antidiabetic properties, there is a need to develop an effective and safe nanoemulsion-based delivery system that could enhance their therapeutic potential as an alternative treatment approach.

1.3 Aims and Objectives

The aim of this study is to develop a lipid-based drug delivery system with efficient bioactivity and safety profile.

1. To synthesize and characterize a lipid nanoemulsion containing combined extracts of propolis and *Nigella sativa*.
2. To evaluate the safety profile in *Sprague dawley* rats.
3. To evaluate the antidiabetic potential of the synthesized nanoemulsion in diabetic rat model.

Chapter 2

Literature Review

2.1 Diabetes

Diabetes is a chronic metabolic disorder characterized by consistently elevated blood glucose levels (hyperglycemia), caused by insufficient production of the hormone insulin from the pancreas, lack of sensitivity of the body cells to insulin and glucose uptake, or a combination of both. According to WHO, diabetes is classified into two main types: Type 1 diabetes and Type 2 Diabetes.

2.1.1 Type 1 Diabetes

Type 1 diabetes is an autoimmune condition in which the beta cells of the pancreas are attacked by auto antibodies, leading to reduction in insulin production. The condition usually presents in early childhood, but can develop at any age. This autoimmune response can be triggered by a few predisposing factors including genetic factors, environmental factors and viral infections [12]. The beta cells of the pancreas are mistakenly attacked and destroyed by the immune system, disrupting the production of insulin, without which the body cells cannot take up glucose from the blood into the tissues, leading to consistently elevated blood sugar levels (hyperglycemia). Lack of insulin also triggers the body to begin breaking down fats for energy, leading to the production of ketones, and ultimately, if left

untreated, a condition called diabetic ketoacidosis. Symptoms of T1DM develop rapidly, due to the body not being able to use glucose efficiently and include polyuria, polyphagia, polydipsia, weight loss, fatigue and blurred vision [1].

2.1.2 Type 2 Diabetes

Type 2 diabetes accounts for 90–95% of global diabetes cases, affecting approximately 462 million people in 2017 [13]. Its rising prevalence is driven by rapid cultural, economic, and societal changes, aging populations, unplanned urbanization, shifts in diet (such as higher intake of processed foods and sugar-sweetened beverages), obesity, reduced physical activity, and unhealthy lifestyle choices.

While T2DM primarily affects adults, its incidence among children and adolescents is growing (American Diabetes Association, 2020). Insulin resistance arises from excess adiposity, which triggers the release of multiple proinflammatory cytokines and adipokines from adipose tissue.

These molecules interfere with insulin signaling pathways in peripheral tissues, leading to impaired glucose uptake from the blood. Initially the pancreatic beta cells try to compensate for this by boosting insulin production, however over time, their function deteriorates, leading to both insulin deficiency and insulin resistance [14].

Chronic Hyperglycemia in T2DM can lead to both macrovascular complications such as cardiovascular disease, peripheral vascular disease and cerebrovascular disease, and microvascular complications such as diabetic nephropathy, retinopathy and neuropathy.

Symptoms include polyuria, polyphagia, polydipsia, sudden changes in weight, excessive hunger, vision problems, increased thirst and urination, slow healing sores, and frequent mood changes [15]. Other complications besides traditional ones (Fig 2.1) are now shown to be associated with long-term diabetes.

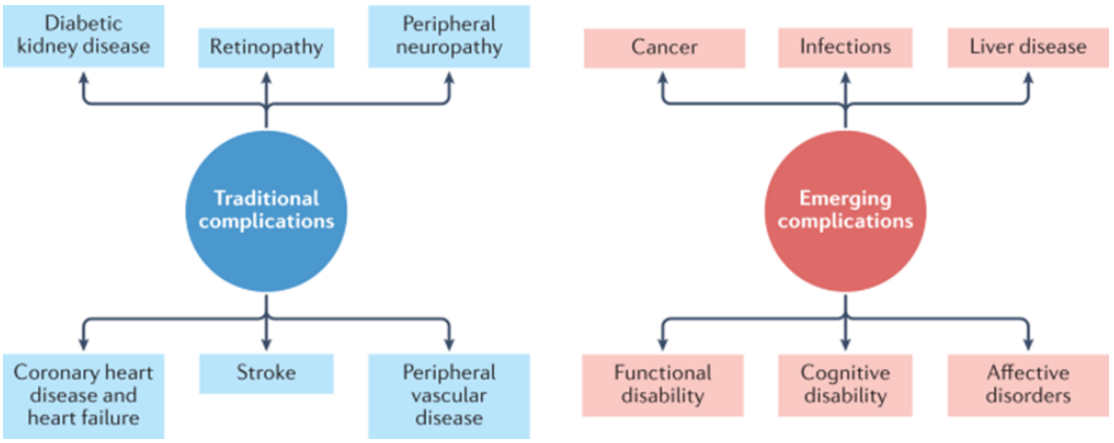

FIGURE 2.1: Complications of Diabetes mellitus [16]

2.2 Diagnosis of Diabetes

The process of diagnosing diabetes, both type 1 and type 2 involve a mix of clinical evaluation combined with laboratory tests. For a diagnosis to be made, the fasting blood glucose levels must exceed 126 mg/dl, and plasma glucose levels must exceed 200mg/dl at the 2hr mark during an oral glucose tolerance test. [17]. For symptomatic patients, a random plasma glucose $\geq$200 mg/dL (11.1 mmol/L) can also support a diagnosis of diabetes. The assessment of types 1 vs 2 diabetes takes into consideration the age at diagnosis of diabetes, the body mass index at diagnosis, presence of autoantibodies, and C-peptide levels in the blood [18].

2.3 Prevalence of Diabetes

Between 1980 and 2014, the number of individuals with diabetes rose from 108 million to 422 million. According to WHO data, diabetes was the direct cause of 1.5 million deaths worldwide in 2019. Patients under the age of 70 accounted for 48% of this burden. The percentage of adults with diabetes was 11.77 percent in 2016, 16.98 percent in 2018, and 17.1% in 2019 [19]. The prevalence of diabetes in Pakistan has been rising annually (fig 2.2). According to estimates from the International Diabetes Federation, there were an expected 33,000,000 cases of

diabetes in Pakistan in 2022, affecting around 26.7% of people [20]. Every year, this trend increases, placing a strain on individuals and health care systems.

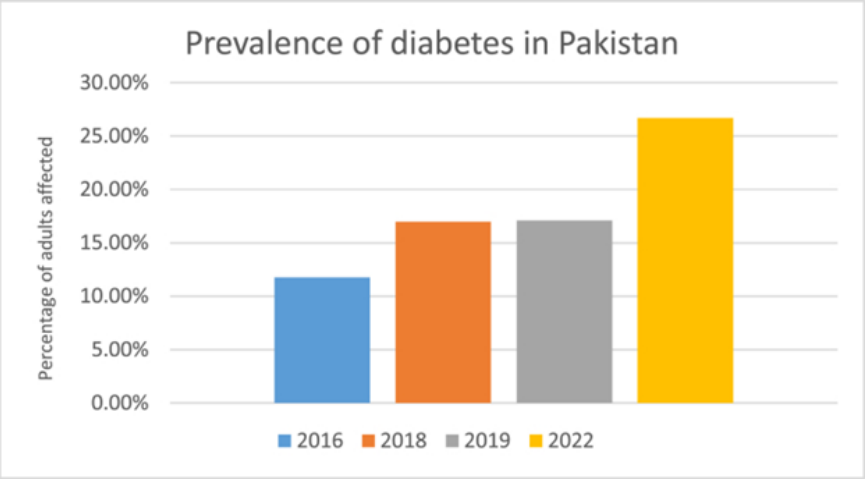

FIGURE 2.2: Prevalence of diabetes in Pakistan [19]

2.4 Drug Therapy for Diabetes

Diabetes can be managed by a range of drugs, some of which increase the secretion of insulin, whereas others increase the sensitivity of the body cells to the hormone. The most widely used biguanide, metformin, decreases the glucose production in the liver, by inhibiting gluconeogenesis. It reduces the gastrointestinal absorption of glucose and increases insulin sensitivity and uptake by the skeletal muscles. This allows it to maintain blood glucose levels, both under fasting and post-prandial conditions, without causing the risk of hypoglycemia [21]. Glimepiride and glyburide are examples of sulphonylureases that function by inducing the release of insulin from the pancreatic beta cells. They accomplish this by blocking the cell membrane's ATP-sensitive potassium channels, which depolarizes the cell and opens calcium channels, which releases insulin [22]. By stimulating peroxisome proliferator-activated receptor-gamma in the liver, skeletal muscles, and adipose tissue, thiazolidinediones, or TZDs, improve insulin sensitivity. This results in decreased hepatic glucose production and increased blood glucose absorption by activating genes related to glucose and lipid metabolism [23].

Dipeptidyl Peptidase-4 (DPP-4) inhibitors are another commonly used group of drugs that work by inhibition the action of DPP-4, an enzyme that breaks down incretin hormones. This leads to an increased amount of incretin hormones within the body, which stimulates the release of insulin from the beta cells of the pancreas and reduces glucagon secretion [24]. Sodium-Glucose Cotransporter-2 (SGLT2) Inhibitors block the SGLT2 proteins in the renal proximal tubules, which leads to reduced glucose reabsorption back into the blood from the urine. This promotes glycosuria, leading to decreased blood glucose levels. They also offer benefits including weight loss and reduced blood pressure. In cases of absolute deficiency of insulin, the hormone must be administered directly. Insulin is used primarily in type 1 diabetes, but also to some extent in type 2. Depending upon the patient's needs, insulin can be rapid acting, immediate acting or long acting, and mimics the actions of physiological insulin, by clearing glucose from the blood and increasing uptake by the body cells [25].

2.5 Side Effects of Synthetic Medications for Management of Diabetes

Conventional management of diabetes using synthetic medications focuses primarily on managing the symptoms of the disease, rather than treating the underlying condition. These medications, whilst effective in many cases, do not come without a range of adverse effects that can, in the long term, result in health complications and reduced quality of life. Furthermore, compliance from patience regarding lifelong treatment regimens poses a significant challenge [25].

Metformin, a first-line class treatment for Type 2 DM, results in gastrointestinal disturbances such as nausea and diarrhea. Rarer adverse effects include lactic acidosis, particularly in patients with renal impairment. Sulfonylureas, known for increasing insulin secretion, carry a risk of weight gain and hypoglycemia, especially challenging for elderly patients or those with irregular eating habits [25].

Insulin therapy, critical for many, is associated with weight gain, hypoglycemia, and local injection site reactions, requiring meticulous monitoring [26].

The necessity of regular injections further impacts patient adherence and quality of life. TZD'S are associated with increase in body weight, retention of fluid and congestive heart failure [25]. This underscores the need for exploration of novel safer alternative treatment options.

2.6 Plant Based Therapeutics

Phytotherapy, or plant-based therapeutics, refers to the use of medicinal plants and their extracts to prevent, cure and alleviate various diseases. This practice, rooted in traditional medicinal systems such as Chinese medicine, Unani and Ayurveda, is gaining popularity in modern healthcare to the growing recognition of the therapeutic potential of plant metabolites, or phytochemicals [27].

These bioactive compounds, produced as a byproduct of plant metabolism include alkaloids, flavonoids, terpenoids and polyphenols, and exhibit a range of therapeutic effects, including antioxidant, anti-diabetic, anti-inflammatory, antimicrobial and even anti-cancer effects.

Moreover, scientific research has validated and proven the therapeutic potential of medicinal plants, leading to the development of standardized phytochemical formulations and supplements that are incorporated into integrative, alternative and complimentary therapies [28].

The appeal of plant-based therapeutics lies not only in their natural source, but also for their potential to cause fewer side effects compared to synthetic medications [29]. A growing body of evidence in now encouraging the integration of phytotherapy into conventional treatment regimes, especially for chronic conditions such as diabetes, where lifelong medication is required [30].

2.7 Propolis

Propolis is a natural adhesive resinous mixture produced by honeybees and is a mixture of plant resins, beeswax and honeybee secretions. The bees mix these plant materials with their saliva and beeswax, creating a sticky, resinous substance used to seal cracks, reinforce hive structure, and maintain sterility within the hive due to its antimicrobial properties. To harvest propolis, beekeepers either scrape it off (fig 2.3) directly from hives, or trap it using specialized mesh sheets [6]. After collecting, it is purified by removing dead bees and wood particles, after which it is processed for various uses. Its therapeutic efficacy is attributed to its balsamic component, which is rich in phytochemicals and generally consists of a mixture of 80-100 different constituents. The specific compounds of propolis are phenolic compounds (flavonoids as main constituents, phenolic acids, and their esters, phenylpropanoids), terpenes and terpenoids, ketones, aromatic aldehydes and alcohols, proteins, fatty acids, waxy acids, amino acids, hydrocarbons, steroids, stilbenes, sugars, vitamins, minerals, and enzymes [31].

FIGURE 2.3: Propolis on beehive (Stock image)

Furthermore, it exhibits great diversity in its therapeutic effect, primarily due to its physiochemical properties which are affected by the bee species that produced it, the geographical location, the climate, season of collection as well as type

of solvent and extract method used to isolate the phytochemicals [32]. Due to variation in phytochemical profile, propolis and its types differ in their properties and therapeutic effects as well [33].

2.7.1 Traditional Uses

Propolis has long been used in folk medicine to treat a range of diseases as an antibacterial, anti-inflammatory and an antifungal agent, as well as to aid wound healing and provide local anesthesia. It has also been used in treatment of cold, cough, flu, asthma and gastric issues. It is still used as an active substance in cosmetics, supplements and even medicinal sweets. The compound itself has no nutritional value but exerts a strong biotic effect and has been used in beverages and foods to boost the immune system [34]. Studies show that its absorbance in the body can be increased by incorporating its hydrophobic compounds within nano drug delivery systems [35].

2.7.2 Pharmacological Properties

Propolis has demonstrated a range of pharmacological effects that make it valuable for treating a range of disorders and has been tested in-vivo and in-vitro to show anti-diabetic, antioxidant, anti-inflammatory and anti-cancer properties. A study reported that a 30% or higher concentration of ethanolic extract of propolis showed antidiabetic potential by lowering blood glucose levels in diabetic rats [36]. Another study with nano encapsulated Egyptian propolis using chitosan showed significant decrease in blood glucose levels of diabetic rats of treated group compared to control [37]. Flavonoid constituents such as apigenin, chrysin, galangin, and pinocembrin contribute to it antidiabetic effect by lowering blood glucose levels. It exhibits anti-inflammatory effects by blocking the NF-κB pathway and modulating TNF-α, IL-6, and IL-1β. It also reduces the production of reactive oxygen species. This is important for type 2 diabetes as subclinical inflammation in major organs of the body leads to insulin resistance and symptoms of hyperglycemia [38].

2.8 *Nigella sativa*

Nigella sativa, also called black cumin, or black seed, is an annual flowering plant, with large fruit, famous for its culinary use as well as benefits for medicinal purposes. Its seed, in the form of oil, paste, powder or extract has been used extensively in traditional medicine to treat asthma, headache, bronchitis, anorexia and hypertension [40]. It is advised for daily usage in prophetic medicine and is regarded by Muslims as one of the best natural therapeutic remedies [41].

These uses stem from their wide array of therapeutic effects, which have been validated scientifically, such as antioxidant, anti-inflammatory, anti-bacterial, cardioprotective and neuroprotective properties. These can be attributed to the presence of bioactive compounds, the most important of which are thymoquinone (30%-48%), thymohydroquinone, dithymoquinone, p-cymene (7%-15%), carvacrol (6%-12%), 4-terpineol (2%-7%), t-anethol (1%-4%), sesquiterpene longifolene (1%-8%) α-pinene and thymol etc. Black seeds also contain some other compounds in trace amounts such as alkaloids, but the pharmacological benefit is mostly attributed to the presence of quinines [40, 42].

2.8.1 Medicinal properties of *Nigella sativa*

The extract of *Nigella sativa* has been studied extensively for its medicinal properties (fig 2.4). It has shown antimicrobial effects against both gram positive as well as gram negative bacteria, *Candida albicans* and species of *Trichophyton* [42]. Its shows antioxidant activity in vitro, successfully reducing levels of pro-inflammatory mediators IL-1, IL-6, TNF-α, IFN-γ and Cox -2, making it a suitable therapeutic option for treatment of arthritis [43].

Emerging research has also highlighted its potential in cancer therapy, where it is able to induce apoptosis and inhibit tumor growth. Moreover, it has also shown gastro-protective, antianxiety, anti-ulcer, cardioprotective and anti-asthmatic effects [40].

FIGURE 2.4: Traditional uses of *Nigella sativa* [43]

The antidiabetic effect of *Nigella sativa* has been tested in vivo and invitro. Studies show that combination of *Nigella sativa* combined with oral antidiabetic drugs, enhances glycemic control by improving pancreatic beta cell function and reducing insulin resistance [44]. A clinical trial showed that levels of HbA1c were reduced in type 2 diabetic patients on receiving 2 grams of *Nigella sativa* twice daily for 1 year, compared to control group. The patients who received Nigella Satvia showed decreased insulin resistance and upregulated beta cell activity. was significantly decreased [44]. Another study showed that the administration of 2 grams of *Nigella sativa* daily for 1 year led to significant reduction of HbA1C [45].

2.9 Combining Multiple Extracts In Polyherbal Formulations

Synergism in phytotherapy refers to the phenomenon where the combined use of multiple extracts from different sources and plants produces a therapeutic effect that is greater than the sum of their individual effects. Each plant contains a unique array of phytochemicals that interact with the bioactive compounds of another extract in a complimentary manner, boosting each other's efficacy whilst potentially reducing unwanted side effects. Furthermore, the presence of a wider

range of bioactive compounds allows a multitargeted approach, with greater therapeutic efficacy and reduction in individual dosage during treatment regimens [9].

Combining extracts of different plants to observe their collective effect is a known scientific method for investigating the best therapeutic options for treatment of diseases and is a known practice of treating diseases in traditional medicine especially Ayurveda [46]. A study involving nanoparticles of a polyherbal formulation using combined extracts of *Momordica charantia*, *Trigonella foenum-graecum*, *Nigella sativa*, and *Ocimum Sanctum* showed greater antidiabetic efficacy in mice in the form of nanoparticles, compared to combined extract alone [47]. Another study involving a polyherbal formulation of 14 herbs showed better antimicrobial efficacy and gastrointestinal pathogens, compared to standard antibiotics [48]. Combination of *Glycosmis pentaphylla*, *Tridax procumbens*, and Mangifera indica in streptozotocin induced diabetic rats showed antidiabetic activity comparable to standard drug glibenclamide at 250 and 500mg/kg respectively [49].

2.10 Lipid Based Nano Drug Carriers

Lipid based nanoparticle formulations are a highly versatile and adaptable class of drug delivery systems that are widely used in research and pharmaceutical industry. They utilize lipids that are biocompatible and offer unique advantages such as small particle size, long term stability, biocompatibility, bioavailability, controlled and sustained release of drug and the ability to encapsulate a wide range of lipophilic and hydrophobic substances. They can be tailored and designed to specifically meet the needs of their intended application [10]. There are many different types of lipid based nano drug delivery systems.

2.10.1 Liposomes

Liposomes are spherical vesicles that consist of a phospholipid bilayer encasing an aqueous core. Hydrophobic substances can be incorporated into the aqueous core and hydrophobic ones into the phospholipid bilayer. Thus, they can be used to

deliver both water and fat-soluble drugs and can be surface modified using ligands to target specific cells [10].

2.10.2 Nanoemulsions

Nanoemulsions are oil within water, or water within oil colloidal systems stabilized with surfactants, formulated using lipids that are liquid at room temperature. For oil within water emulsions, the oil droplets are broken down to a nano scale and suspended in an aqueous medium and can encapsulate lipophilic drugs. Nanoemulsions can be delivered through a range of routes including oral, IV and topical administration [50].

2.10.3 Solid Lipid Nanoparticles

Solid lipid nanoparticles are formulated using lipids that exist in solid form at room temperature such as stearic acid and palmitic acid. They consist of a solid lipid core encasing hydrophobic and lipophilic drugs and exist in solid form at both room and body temperature. They offer many advantages such as biocompatibility, protection of the drug against harsh external environment, sustained drug release and biodegradability. However, they are difficult to isolate and require temperature sensitive techniques for synthesis. Furthermore, due to the crystalline nature of the lipids, they undergo phase changes during storage which causes leeching out of drug [51].

2.10.4 Nanostructured Lipid Carriers

Nanostructured lipid carriers are a modification of solid lipid nanoparticles and consist of a combination of solid liquids and liquid oils. They have a less ordered and crystalline structure in their core due to the incorporation of two types of lipids, which allows higher drug loading and less drug loss during storage. They also increase the drug solubility in the matrix and provide a slower release of drugs compared to solid lipid nanoparticles [52].

2.10.5 Lipid Polymer Hybrid Nanoparticles

Lipid Polymer Hybrid Nanoparticles are a combination of both lipid and polymer. The lipid forms the outer shell, providing biocompatibility and drug retention, whereas the polymer core provides structural integrity and slower drug release. The outer shell can be functionalized using various molecules and ligands that allow target specific drug delivery [53]. Lipid polymer hybrid nanoparticles (fig 2.5) offer the advantage of high stability, high drug loading, elevated drug half-life and sustained drug release [54]. Due to their beneficial properties and the ability to manipulate and optimize their structure to meet specific needs, lipid based nano drug formulations have been used extensively in research to explore their therapeutic effect (table 2.1).

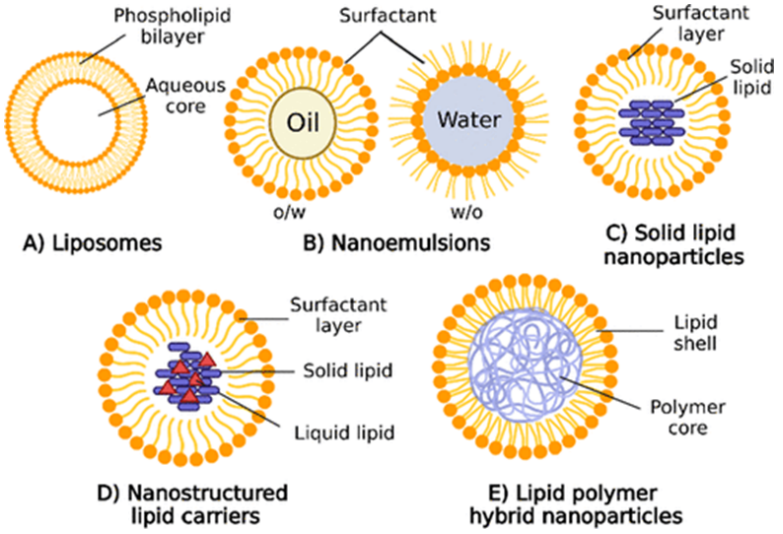

FIGURE 2.5: Lipid based nano drug delivery systems [54]

TABLE 2.1: Therapeutic uses of lipid based nano drug delivery systems

Type of lipid nano drug delivery system	**Active ingredient**	**Purpose**	**Findings**	**Ref**
Liposome based formulation of anti Alzheimer drug	Donepezil	Tested in cell lines	Showed effective intracellular delivery of drug and ability to overcome blood brain barrier.	[55]

Continued to next page

Table 2.1 (continued from previous page)

Type of lipid nano drug delivery system	Active ingredient	Purpose	Findings	Ref
Dorzolamide Hydrochloride loaded nanaoemulsion	Dorzolamide Hydrochloride	Eye drops for glaucoma treatment	Higher therapeutic efficacy, quicker onset of action, and longer effect compared to drug solution	[56]
Nintenab loaded solid lipid nanoparticles	Nintedanib	Treatment for idiopathic pulmonary fibrosis	Tested in vivo in rat model. Showed 2.87-fold increase in bioavailability and improved lung function mechanics	[57]
Nanostructured lipid carriers loaded with miltefosine	Miltefosine	Therapeutic effect against Cutaneous leishmaniasis	Tested in mice. Showed 2.5 folds increase in anti-leishmanial efficacy and slower drug release compared to control.	[58]
Lipid polymer hybrid nanoparticles	CRISPR/Cas9 plasmids	Gene therapy for drug resistant glioblastoma	Tested in mice. Showed enhanced therapeutic effect, inhibited tumor growth and prolonged survival of tumor bearing mice.	[59]

2.11 Nanoemulsion

Emulsions are colloidal dispersions consisting of two immiscible liquids, usually oil and water. Under normal circumstances, these two components would not mix resulting in phase separation of the solution. However, in emulsions, one liquid is dispersed in droplet or globule form in another liquid and stabilized using surfactants [50]. The surfactant serves to reduce the surface tension between the two liquids and prevents particles from coalescing resulting in a stable mixture (fig 2.6). Nanoemulsions are emulsions with a droplet size ranging from 20-500nm

generally, with their size providing them unique properties. They are extensively used in the food, pharmaceutical and cosmetics industry [50, 60].

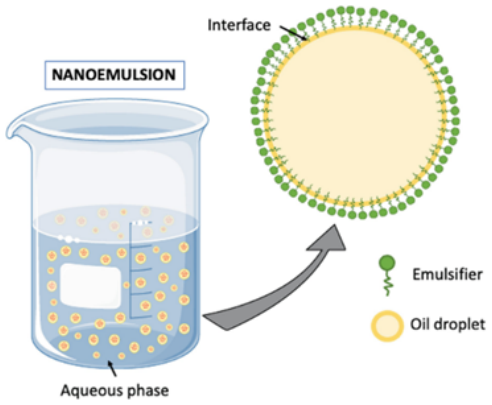

FIGURE 2.6: Nanoemulsion [60]

2.12 Type of Lipid Nanoemulsions

Water in oil emulsion consists of small droplets of water that scattered within the oil phase and stabilized using surfactants (fig 2.7). These emulsions provide protection for water soluble compounds and maintain stability in lipophilic environments. The surfactants, such as sorbitan esters, have a low hydrophilic-lipophilic balance (HLB), which indicates that they have a stronger affinity for the lipid or oil phase [50].

Oil in water emulsion consists of small droplets of oil that are scattered within the aqueous phase and stabilized by surfactants (fig 2.8). These surfactants, such as tween 80, have a high hydrophilic-lipophilic balance (HLB). They coat the oil particles and decrease the surface tension between the water and oil phase thus preventing aggregation and phase separation and enhance the stability of the emulsion. The are ideal for delivering hydrophobic drugs encased within the oil droplets [61].

Water-in-oil-in-water (W/O/W) or oil-in-water-in-oil (O/W/O) emulsions are examples of double emulsions, which are more complicated systems made up of an emulsion inside an emulsion. The first internal emulsion must be formed first, and then the second emulsion that surrounds it must be formed. These emulsions are

made in two steps. They offer the benefit of shielding delicate substances with additional layers of defense while regulating how quickly they are released into biological systems. [62].

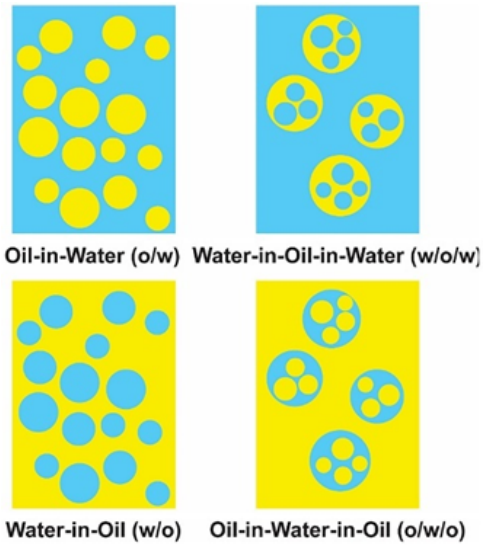

FIGURE 2.7: Types of emulsions [63]

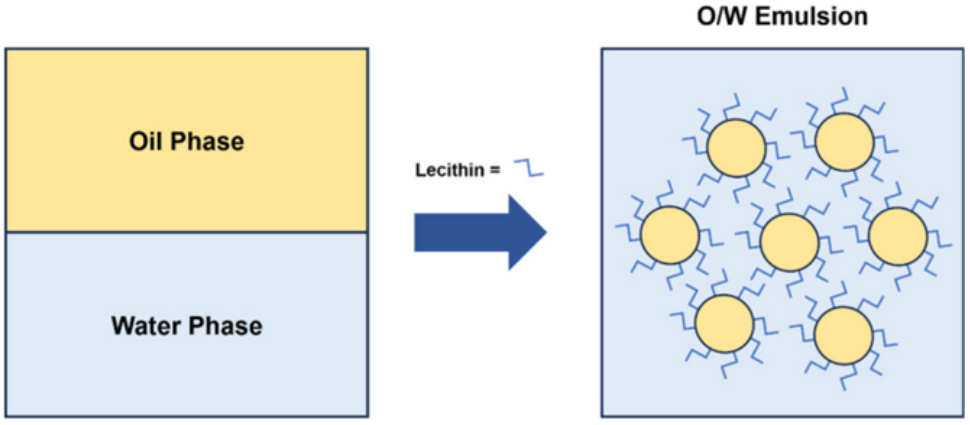

FIGURE 2.8: Stabilization of emulsion using lecithin as a surfactant [64]

2.13 Components of Lipid Nanoemulsion

2.13.1 Oils

The oil phase consists primarily of lipids that serve as the carrier for active ingredients within a lipid nanoemulsion and are liquids at room temperature. Oils used are primarily medium chain triglycerides, long chain triglycerides and essential

oils. Selection of the lipid is based upon the intended use of the nanoemulsion, the compatibility of the lipid with biological membranes and the solubility of the active ingredient within the oil [61]. To create a stable emulsion, the chosen lipid must be very compatible with the surfactant; its polarity, water miscibility, interfacial tension, and viscosity can change the stability and droplet size of nanoemulsions. [50].

Many lipids have been used to formulate nanoemulsions. Oleic acid is a monounsaturated omega-9 fatty acid commonly found in natural oils such as olive oil, avocado oil, peanut oil and animal fats. It shows biocompatibility within biological systems and also possesses antioxidant and anti-inflammatory properties. Within nanoemulsions oleic acid interacts with surfactants to encapsulate both lipophilic and hydrophobic drugs, thus providing a stable emulsion with a sustained release profile. Literature also shows that oleic acid itself can improve glycemic control, by reversing the inhibitory effect of inflammatory cytokines on insulin production [65].

Other oils used in nanoemulsions include MCT oils and essential oils. Medium chain triglyceride oils consist of a mixture of triacylglycerols, that are rich in medium chain fatty acids and can be obtained from coconut and palm oil. They are ambient at room temperature, have very low water solubility that prevents Ostwald ripening and result in stable emulsions with a smaller particle size [66]. Essential oils are volatile substances which are extracted from aromatic plants and within a nanoemulsion provide a dual role of providing both structural integrity as well as therapeutic effects. They provide antioxidant, antimicrobial and anti-inflammatory effects and prevent the active ingredients from degradation [67].

2.13.2 Surfactants

Surfactants are amphiphilic molecules, with a hydrophobic and hydrophilic portion within their molecular structure, and this hydrophilic-lipophilic balance is represented by HLB value. The primary role of a surfactant within a nanoemulsion is to decrease the surface tension between the oil and water phase [61]. During the

formation of a nanoemulsion, surfactant molecules rapidly gather and adsorb at the oil-water interface, where they reduce the energy required to break down the particles into smaller sizes. They surround the oil particles with their hydrophilic portion pointing outwards towards the water phase. This stabilizes the oil droplets and prevents their coalescence, preventing phase separation of the oil and water mixture [50].

The concentration of surfactants with a nanoemulsion can range from 1 to 10%, and has a great effect on the droplet size. Insufficient surfactants will lead to phase separation as there is not enough to stabilize the oil droplets inside the water phase. A higher concentration can result in the formation of micelles within the system that can interfere with the stability and therapeutic effect of the emulsion [63]. The components of nanoemulsion-based systems, particularly surfactants, raise concerns regarding toxicity. When consumed in large quantities, surfactants can lead to gastrointestinal issues, while topical application may result in skin irritation [68]. Therefore the proper selection of surfactants (fig 2.9) is essential. Ideally, the minimum amount of surfactant should be used, especially in orally administered formulations.

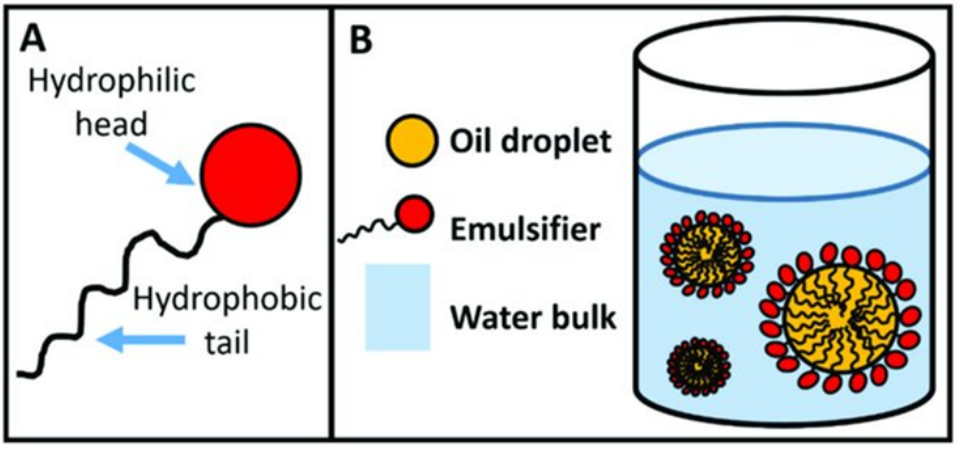

FIGURE 2.9: Role of surfactant within an emulsion [69]

Ionic surfactants such as sodium laryl sulphate, can carry a positive or negative charge, which provides strong electrostatic repulsion between the oil droplets, keeping them apart. Non-ionic surfactants include sorbitan esters such as spans

and polysorbates such as tweens. They do not ionize in solution and provide stability to the emulsion through steric hindrance. Due to their non-ionic nature, they are more resistant to changes in electrolytes and pH [63].

Tween 80 also referred to as polysorbate 80, is a non-ionic surfactant frequently used in food and pharmaceutical industry. Its molecular structure features a hydrophilic head group composed of polyethylene oxide units and a lipophilic tail from oleic acid, allowing it to effectively reduce interfacial tension and provide steric stabilization at the oil-water interface. Polysorbate 80 has been linked to a drop in blood pressure in animal studies. Drugs formulated using the compound have shown increased intestinal absorption and uptake into the brain and plasma. However, it has also been linked with several undesirable effects. Within food, even low levels of undigested polysorbate 80 can promote the movement of bacteria across the intestinal epithelium. Within drug formulations it has been implicated in hypersensitivity, anaphylaxis rash as well as injection site reactions, with several studies reporting cases of liver and renal toxicity [70].

2.13.3 Stabilizers

Stabilizers provide long term stability to a nanoemulsion, by preventing Ostwald ripening, droplet flocculation, and coalescence during storage. They gather at the interface of oil and water and provide a mechanical barrier and provide steric stabilization through their structure [63]. They are used frequently in combination with other surfactants to improve the stability of an emulsion. Soy lecithin is a natural emulsifier derived from soybeans. Emulsions using soy lecithin in combination with other surfactants have been used widely to deliver antimicrobial agents and drugs as well as transdermal delivery of anti-cancer drugs [71].

2.13.4 Active ingredient

Active ingredients incorporated into nanoemulsions include vitamins, plant extracts, pharmaceutical drugs, antibiotics and bioactive molecules. Lipophilic molecules

can be incorporated into the oil phase whereas hydrophilic ones can be incorporated into the aqueous phase. The loading of these active molecules into nanosized globules not only protects them from enzymatic degradation within the body but also provides improved absorption into the bloodstream and enhanced cellular uptake, all of which can enhance the therapeutic efficacy [50]. By being encapsulated within these oil/water droplets, the drug is released slowly providing a sustained released profile. The amount of drug incorporated depends on its affinity for the oil and water phase along with the concentration of surfactants and lipids used [63].

Research shows successful incorporation of a range of drugs into nanoemulsions to improve their therapeutic efficacy, including doxorubicin, to ensure safe an effective delivery to cells for antitumor activity [72], plumbagin to test its antiproliferative effect on prostate cancer cells [73] and zolmitriptan to increase its bioavailability within the brain to help treat migraines [74]. In addition, nanoemulsions of plant extracts have been developed to enhance their bioavailability including those of curcumin [75] and *Lawsonia inermis* [76]. Furthermore, nanoemulsions to improve the bioavailability of vit D have also been developed [77].

2.14 Methods of Preparation

Methods to synthesize nanoemulsions can be divided into high energy and low energy methods. High energy methods are used to prepare nanoemulsions on a larger scale. They apply mechanical forces to break down the droplets within the emulsion to a nanoscale [63]. Low energy methods depend on the changes of components of the system and its surroundings to produce the emulsion. Surfactants are crucial in both techniques because they lower the surface tension at the oil-water interface, which facilitates the use of shear forces to break up the droplets and stabilizes them inside the emulsion [61]. The choice of method depends upon the properties of the oil and surfactant used as well as the intended use of the emulsion. The method used has a great effect on the droplet size as well as the stability [50].

2.14.1 High Energy Methods

High energy methods use mechanical equipment, such as homogenizers or mechanical stirrers to supply the system with a high amount of energy, which generates shear forces that break down the oil and water interface to generate nano sized droplets. High energy methods include high pressure homogenization, ultrasonication and micro fluidization [50].

High pressure homogenization involves forcing the course emulsion, consisting of oil and water phase through a narrow orifice at extremely high pressure (500 to 5000 psi), which exposes the emulsion to intense shear forces and cavitation, breaking the droplets to a nanoscale size (fig 2.10). The droplet size depends upon the homogenizer type, pressure used, the number of passes of the emulsion through the homogenizer, as well the concentration and type of surfactant [50]. Enough surfactant must be present within the emulsion to fully coat and stabilize the particles that are being produced. High pressure homogenization is effective for generating emulsions with a uniform droplet size and is extensively employed in the pharmaceutical sector. However, the method uses a high amount of energy and can lead to temperature increase of the emulsion which can degrade the active ingredients [61].

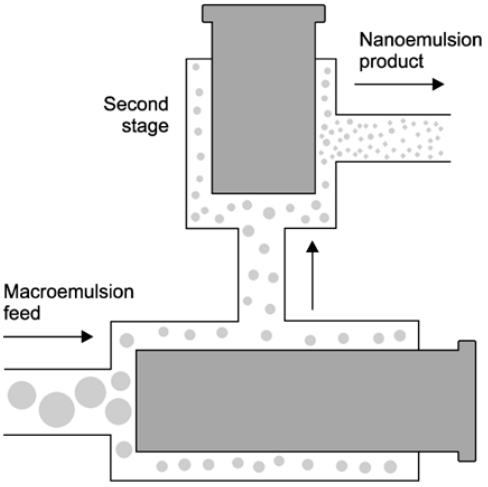

FIGURE 2.10: Homogenization method for preparation of nanoemulsion [78]

High speed homogenization uses rotor systems such as high-speed homogenizers or mechanical stirrers at an RPM of up to 12000 to break down the emulsion

into nanosized droplets using turbulence, stress and shear forces. The droplet size depends on the number of cycles used, the composition of the emulsion, the temperature of the system and the amount of surfactant used. Generally, the synthesis process needs to be optimized to generate smaller droplet size [78].

Ultrasonication uses high frequency ultrasonic waves (fig 2.11) to generate cavitation bubbles within the emulsion, which collapse and generate localized areas of stress and pressure which reduce the droplet size. This method is better than high energy methods in terms of operation and cleaning [50]. Ultrasonication can be achieved using a probe sonicator which delivers sound waves into the emulsion directly through its tip, or through a bath sonicator which generates ultrasonic waves and transmits them through a water bath. Bath sonicators offer a gentler approach and are suitable for much larger volumes [78].

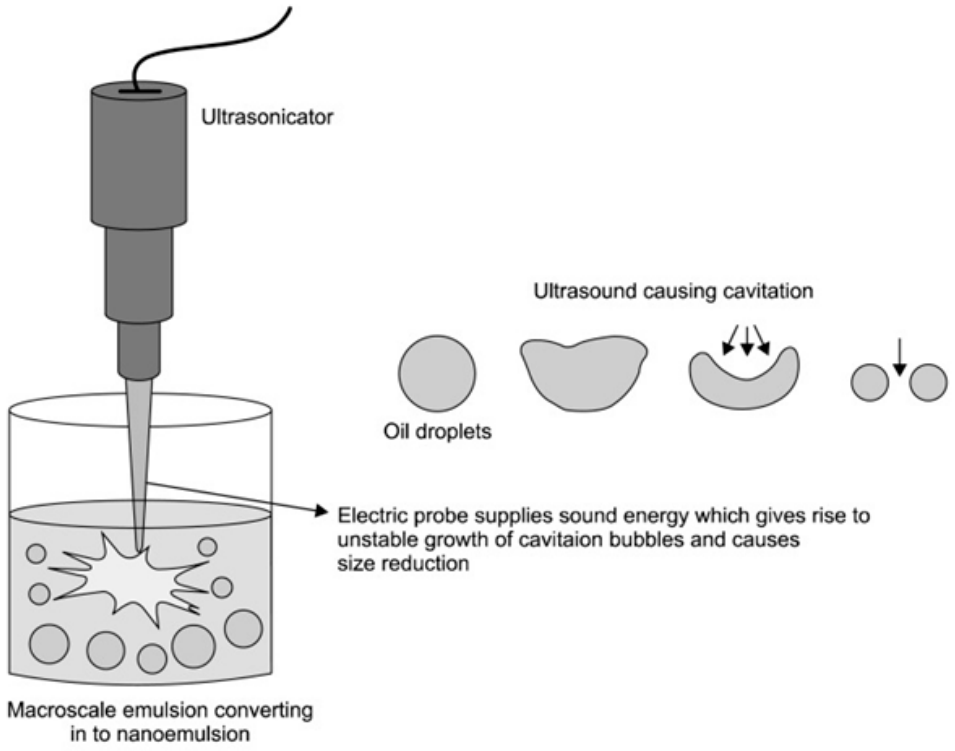

FIGURE 2.11: Ultrasonication method for preparation of nanoemulsion [78]

Microfluidization is another technique which uses a special instrument called a microfluidizer (fig 2.12). The course emulsion is prepared and forced through narrow micro sized channels under high pressure (500 – 20,000 psi). The streams enter an interaction chamber where two streams of the course emulsion collide with

each other at high velocity [63]. The impact generates shear forces that break down the emulsion into nano sized droplets. The process is repeated multiple times to attain the required particle size. Droplet size increases as the homogenization pressure, surfactant concentration and number of processing passes increase. [78].

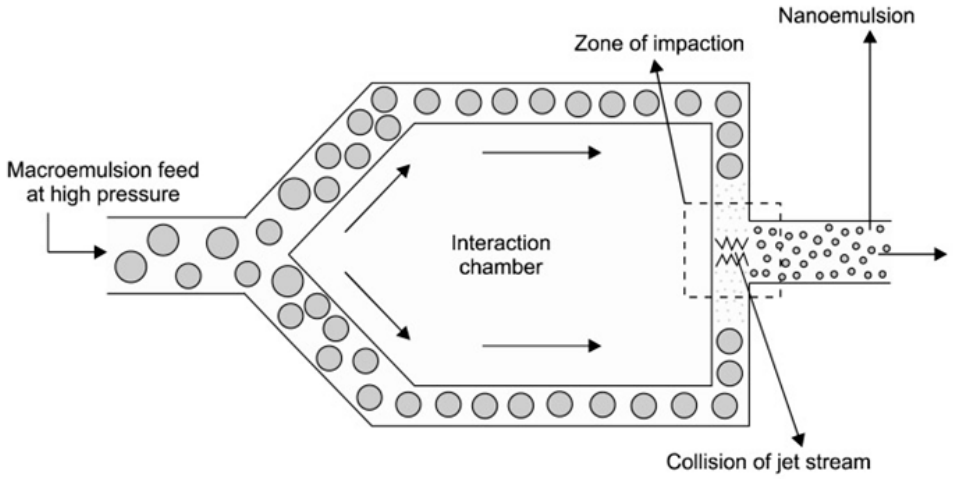

FIGURE 2.12: Microfluidation method for preparation of nanoemulsion [78]

2.14.2 Low Energy Methods

Low energy methods take advantage of the physiochemical properties of the components of the emulsion and their thermodynamic interactions, without requiring the need for high energy mechanical forces. They use simple gentle stirring and offer a more cost effect approach to synthesizing nanoemulsions [63].

Phase inversion temperature method is one such method based on the temperature dependent behavior of non-ionic surfactants. At lower temperatures they are more hydrophilic, favoring water in oil emulsions, but at higher temperatures they become more lipophilic, favoring oil in water emulsions. At the emulsion is heated, dehydration of the surfactant molecules occurs [50]. The emulsion then reaches a bicontinuous (fig 2.13) phase at the phase inversion temperature upon which the system changes from oil in water, to water in oil or vice versa. This results in spontaneous formation of small droplets which become stabilized as the system is rapidly cooled. This method is cost effective but requires careful observation and delicate changes in temperature to reach the optimal particle size [78].

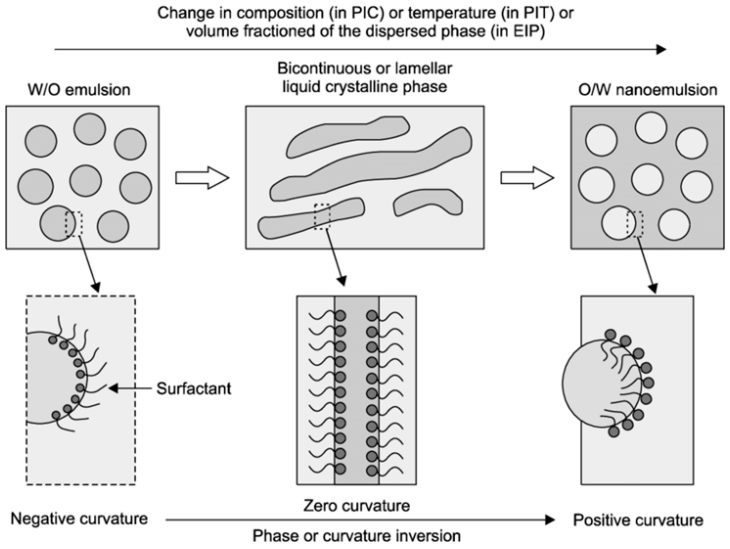

FIGURE 2.13: Phase inversion temperature method for preparation of nanoemulsion [78]

Spontaneous emulsification method involves mixing a surfactant rich oil phase with an aqueous phase. When the two phases interact, the surfactant molecules rapidly diffuse into the aqueous phase, which creates turbulence and generates nano sized droplets. This method does not require any external sources of energy but uses high surfactant and low lipid concentration [50].

The emulsification inversion point method also involves inversion of emulsion type but is achieved by slowly changing the composition of the mixture. Water is slowly added to the oil phase, until it triggers phase inversion, and the system changes from water in oil to oil in water. At this inversion point, the surfactants ability to stabilize both phases reaches a balance which results in nano sized droplets [79]. This method does not require sensitive changes in temperature and is suitable for thermally sensitive formulations (fig 2.14).

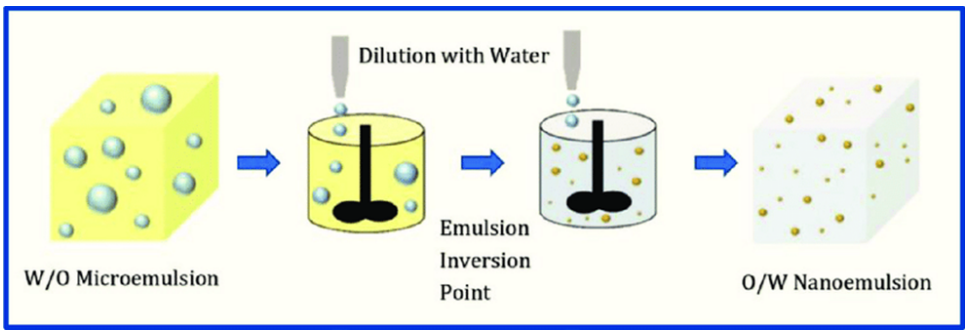

FIGURE 2.14: Emulsification inversion point method [79]

2.15 Stability of Nanoemulsion

The stability of a nanoemulsion is an important factor that determines its efficacy and shelf life. Nanoemulsions are thermodynamically unstable, but kinetically stable systems.

Destabilization kinetics are very slow, hence nanoemulsions can stay stable for up to a few months. If the composition of the solution is not ideal, or if the emulsion is not stored properly, it may become turbid, or cloudy, due to phase separation, flocculation, Oswald ripening or coalescing of the particles [80].

Oswald ripening occurs when smaller particles migrate towards larger particles through the continuous phase, and coalescence occurs where there are not sufficient repulsive forces between the particles leading to particles aggregating together. In addition, the encapsulated drug may start leeching out. Factors that influence the long-term stability include droplet size, choice of surfactant and co-surfactant, zeta potential and storage temperature [81].

2.16 Characterization of Nanoemulsion

Nanoemulsions can be characterized by a range of techniques, which provide us with valuable information regarding their physical and chemical properties. Droplet size and distribution of the particles can be achieved through dynamic light scattering. A laser beam is shone onto the sample and the scattering of light gives information about the distribution as well as size of the particles [82].

Many studies also provide value of zeta potential, which measures the electrostatic charge on the surface of the droplets. This gives an indication of stability, as the higher the charge, the more repulsion will exist between the particles, and they will not coalesce. A higher zeta potential in either positive or negative value indicates good stability of the emulsion [83].

The pH value measures the hydrogen ion concentration within the emulsion. This is critical for compatibility and stability particularly for cosmetic or pharmaceutical applications. Severe changes in pH may indicate chemical instability or degradation. Furthermore, depending upon the intended purpose and route of administration, the pH of the emulsion must be optimized [50]. To determine the stability of the emulsions, visual changes are observed over a specified period, and phase separation, flocculation, creaming and color changes are noticed. Moreover, the emulsions are subjected to different temperatures (4^{o}C, 25^{o}C, 40^{o}C) and closely observed for phase separation. Emulsions may be transparent to milky (fig 2.15), and any instability will result in visual changes. This is important as emulsions, especially those intended for pharmaceutical use, must be able to withstand temperature fluctuations during storage [84].

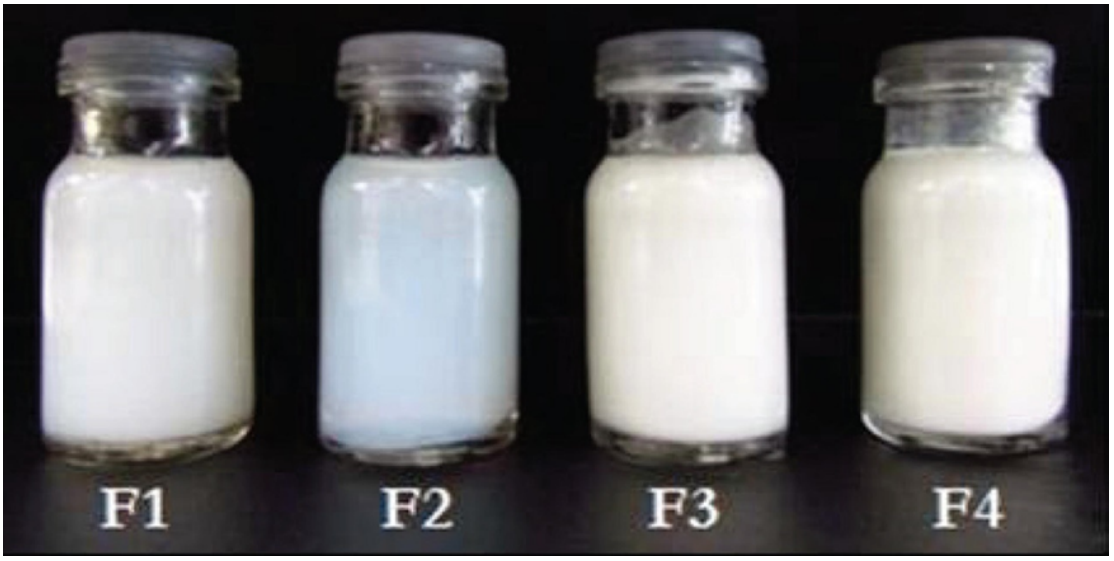

FIGURE 2.15: Low energy nanoemulsions prepared using emulsification method [84]

2.17 Therapeutic Uses of Nanoemulsions

Nanoemulsions have gained significant attention in drug delivery due to their ability to enhance the solubility, stability, and bioavailability of therapeutic compounds, which makes them effective for drugs that are not that soluble in water. Their small droplet size helps to improve absorption, rapid cellular uptake, and

prolonged circulation time in the body. These nano-sized emulsions facilitate targeted and controlled drug release, ensuring a sustained therapeutic effect while reducing the frequency of dosing and minimizing systemic toxicity.

Their biocompatibility and ease of formulation with both hydrophilic and lipophilic drugs make them suitable for a variety of applications, including oral, topical, intravenous, and pulmonary drug delivery. Table 2.2 highlights some recently used nanoemulsions along with their therapeutic effect.

TABLE 2.2: Therapeutic uses of nanoemulsions

Formulation	**Drug**	**Therapeutic use**	**Findings**	**Ref**
Cyclosporin nanoemulsion	Cyclosporin	Treatment for dry eyes	Patients treated with nanoemulsion showed greater and faster improvement compared to control group.	[85]
Nanoemulsion based gel	Piroxicam	Anti-inflammatory effect	Showed good physiochemical properties and enhanced 2.41 times increase in bioavailability compared to commercial formulation.	[86]
Quercetin nanoemulsion	Quercetin	Antidiabetic effect	Showed stability for 45 days, with significant antidiabetic effect and greater bioavailability compared to control.	[87]
Syzgium aromaticum essential oil nanoemulsion	Syzgium aromticum essential oil	Antimicrobial, antibiofilm and anticancer effect	Showed significant antimicrobial, antibiofilm and anticancer effects and induced apoptosis in breast cancer cells.	[88]
Osthole loaded nanoemulsion	Osthole	Intranasal administration for treatment of Alzheimer's	Increased bioavailability of drug using the nanoemulsion with neuroprotective effects	[89]

2.18 Drug Testing in Animal Models

Sprague dawley rats are commonly used in research on drug toxicity and anti-diabetic effects because they are large, easy to handle, and have a calm nature. Their size allows for accurate drug dosing and administration, making experiments more precise. These rats are also well-studied in diabetes research since they develop the disease in a predictable way, showing symptoms like high blood sugar, insulin resistance, and changes in fat metabolism. This consistency makes them ideal for testing new treatments for diabetes [37]. Another advantage of using *Sprague dawley* rats is that their larger organs make dissection and analysis easier. Researchers can examine the liver, kidneys, and pancreas to check for any harmful effects of a drug.

This is especially important in preclinical studies, where both the effectiveness and safety of a drug must be evaluated. Because of these benefits, *Sprague dawley* rats are widely used to study new medications, particularly those for metabolic diseases like diabetes. Their size also facilitates ease of dissection and removal of major organs for histopathological examination.

2.18.1 Disease Induction by Streptozotocin

Streptozotocin is a naturally occurring alkylating agent that was originally discovered in the bacteria *Streptomyces achromogenes*. It is a nitrosourea derivative with the molecular formula $C_8H_{15}N_3O_7$ (fig 2.16) and a molecular weight of 265.22 g/mol. STZ is composed of a glucose moiety linked to a methyl nitrosourea group, which is responsible for its alkylating and cytotoxic properties. Studies conducted on the compound led to the discovery that it is selectively toxic to the beta cells of the pancreas and therefore is used for disease induction in animal models. The freeze-dried powder is kept at -20^{o}C and is pale yellow in color. It is highly soluble in water but unstable in aqueous solutions, particularly at neutral or alkaline pH, necessitating preparation in acidic buffers like citrate buffer (pH 4.5) immediately before use to maintain its activity [91].

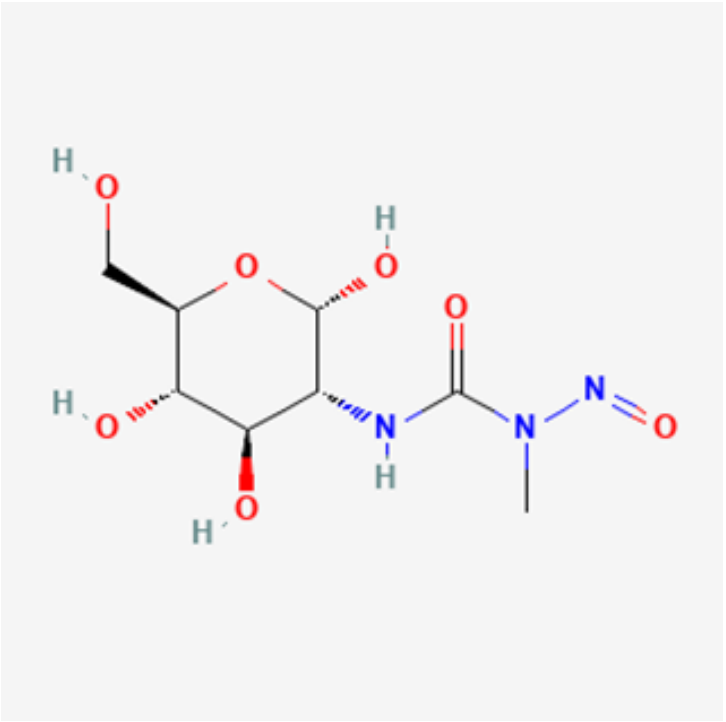

FIGURE 2.16: Molecular structure of streptozotocin [90]

Due to its structural similarity with glucose, STZ is preferentially taken up by the beta cells of the pancreas by the glucose transporter-2 (GLUT2). Once inside the cells, the chemical exerts its toxic effect through several interrelated mechanisms [90]. It acts as an alkylating agent and targets guanine residues within DNA, resulting in single and double stranded breaks. The repair mechanisms generated by the cell lead to energy depletion and cell death. Furthermore, it generates reactive oxygen species which damage DNA, lipids and proteins leading to necrosis and apoptosis. Furthermore, it triggers a generalized inflammatory response in the pancreas intensifying beta cell destruction. This eventually leads to insulin depletion, hyperglycemia and diabetes induction [91].

Streptozotocin solution is typically prepared fresh in cold sodium citrate buffer at a pH of 4.5-5 to ensure stability [91]. The solution is used immediately after preparation and it's administered intraperitoneally, due to its consistent delivery and ease of administration, although IV route can be used as well. Before administering the dosage, rats are placed on 8-12 hour fasting to enhance uptake of STZ by the beta cells. A single dosage of 60mg/kg/b.w or multiple low doses at 30-45/kg/b.w for 3-5 consecutive days can be used to induce diabetes. Blood

glucose levels are measured 24-48 hours after administration and values greater than 200mg/dl are considered an indicator of successful induction [92].

2.18.2 Disease Monitoring and Evaluation

Body weight is an essential parameter in diabetes research as well as toxicity studies as it provides information regarding nutritional status, metabolism and overall health. Monitoring the weight of animals helps evaluate the progression of the disease as well as effectiveness of the treatment, with sudden fluctuations or drops in weight considered as red flags. Blood glucose levels in anti-diabetic studies are the most crucial parameters for assessing effectiveness of treatment. In the case of mice or rats, blood samples can be taken from the tip of the tail (fig 2.17). This causes minimum discomfort to the animal [93].

FIGURE 2.17: Blood collection from tail tip to measure glucose levels [93]

Uncontrolled diabetes can lead to diabetic nephropathy, in which the kidneys are directly affected by the persistent hyperglycemia and oxidative stress, leading to glomerular damage. Key renal markers such as BUN, serum creatinine and albumin are measured at the end of the study period. An increase in BUN and serum creatinine indicates impaired kidney function, while elevated levels of urine albumin (albuminuria) are a sign of early kidney damage, as the kidneys lose their ability to filter out large molecules like proteins [94]. Liver function tests, on the

other hand provide us with an estimate regarding the toxicity of a drug. Key biomarkers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, and albumin levels indicate hepatocellular injury, cholestasis, and liver dysfunction. Elevated ALT and AST suggest hepatocellular damage, while increased ALP and bilirubin reflect bile duct injury or impaired liver function [95].

Histopathological examination involves microscopic analysis of tissue sections to assess structural damage and identify disease-specific changes. In diabetes, tissues like the pancreas, kidneys, liver, and heart undergo significant alterations that can be visualized through histopathological analysis. In the pancreas, STZ-induced diabetes causes β-cell destruction and islet atrophy, which can be observed through staining [96]. Within the liver, drug toxicity results in findings such as areas of necrosis, degeneration and infiltration of inflammatory cells [97].

Chapter 3

Methodology

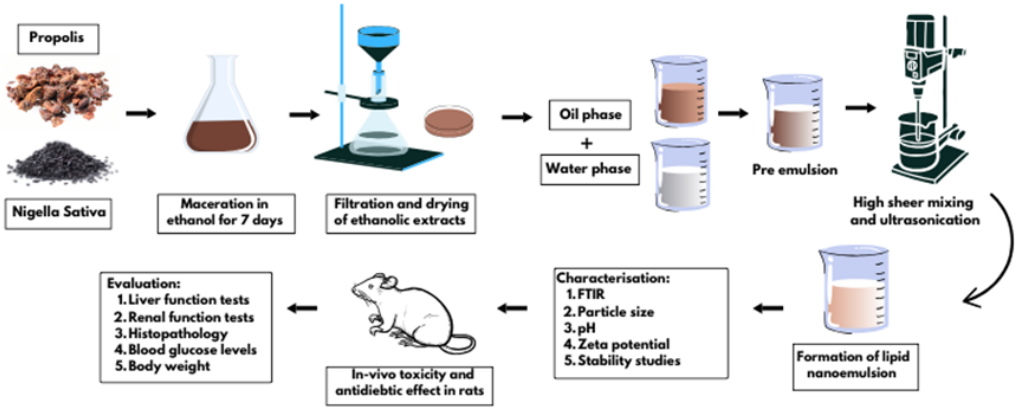

FIGURE 3.1: Overview of research methodology

3.1 Ethical Considerations

The ethical review committee of the Department of Bioinformatics and Biosciences, Capital University of Science and Technology, Islamabad, Pakistan approved the research titled "Development, Safety Evaluation, and Antidiabetic Activity of Propolis-*Nigella sativa* Nanoemulsion".

All procedures followed the committee's guidelines to ensure the ethical treatment of animals, with measures in place to minimize any discomfort or stress to the rats during the experiment.

3.2 Materials Used

Raw propolis from *Apis mellifera* was obtained and verified by Entomology Department at Arid Agriculture University Rawalpindi and commercial grade *Nigella sativa* seeds were purchased from local market (fig 3.2). Tween 80 and Oleic acid were obtained from Croda, and Soybean lecithin was procured from Food Kemiya Pvt Ltd. Ethanol, streptozotocin, sodium citrate dihydrate and citric acid were obtained from Sigma Aldrich. Sterile injection water for preparation of buffer was obtained from local pharmacy.

3.3 Preparation of Extracts

Propolis extract was prepared by drying and crushing 200g of propolis. This was then added to 1000 ml of 70% ethanol in the ratio of 1:5 [98]. The mixture was stirred for 3 hours then macerated for 7 days at room temperature in the dark, with periodic stirring throughout. The solution was then filtered using filter paper (fig 3.3) and ethanol was evaporated using rotary evaporator with water bath set to 50 degrees until a thick viscous sticky extract was obtained. The final extract was then stored in an airtight container at 4 degrees for further use [99].

100g of *Nigella sativa* seeds were ground to obtain a course powder. This was then added to 250ml of ethanol and macerated for 7 days with occasional stirring [98]. The solution was filtered, poured into sterile petri dishes and placed under a fume hood to evaporate the ethanol. A dark brown colored extract was obtained which was collected and stored at 4 degrees until further use.

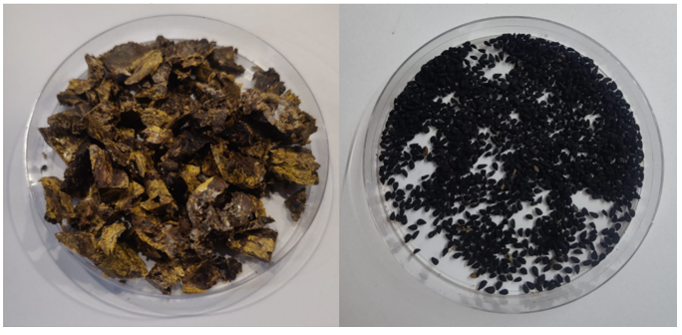

FIGURE 3.2: Dried propolis and *Nigella sativa* seeds

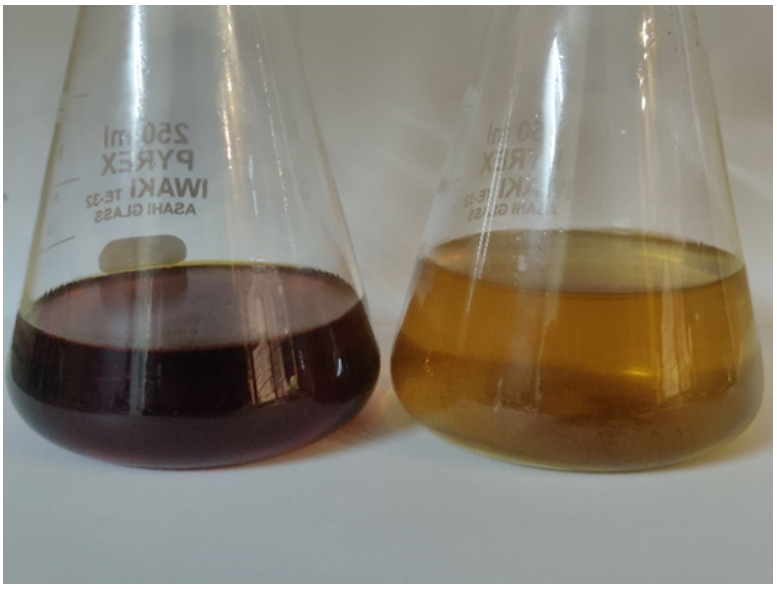

FIGURE 3.3: Ethanolic extracts of Propolis and *Nigella sativa*

3.4 Characterization of Extracts

Characterization of the extracts was performed using Fourier Transform Infrared spectroscopy, with wavelengths set from 400-4000 cm^{-1} and baseline correction conducted using Origin Pro. The purpose was to identify the functional groups and confirm the chemical composition of the propolis and *Nigella sativa* extracts [100].

3.5 Synthesis of Nanoemulsion

The nanoemulsion of propolis and *Nigella sativa* was prepared using two phases. The oil phase consisted of synthetic oleic acid, soy lecithin and the extracts, and the aqueous phase consisted of Tween 80 and double distilled water (fig 3.4). The formulation process was optimized to achieve an ideal particle size, which was determined through utilization of different methods of preparation and varying lipid and surfactant ratios. The formulation exhibiting the best particle size was selected for characterization and subsequently administered to rats. Table 3.1 summarizes the components within the emulsion and their intended purpose.

TABLE 3.1: Components of the nanoemulsion with their intended purpose

Ingredient	**Purpose**
Oleic Acid	Oil phase, solubilizes lipophilic compounds
Soy Lecithin	Surfactant and stabilizer, stabilizes the emulsion
Propolis Extract	Provides bioactive compounds
Nigella sativa Extract	Contributes antioxidants and bioactive compounds
Tween 80	Surfactant, stabilizes the emulsion
Double Distilled Water	Aqueous phase, facilitates dispersion of droplets

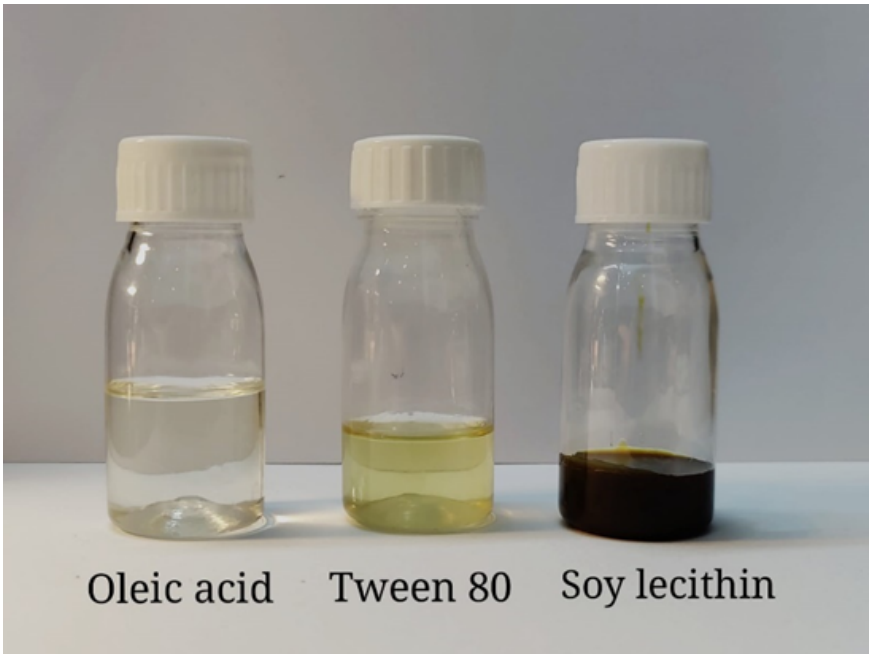

FIGURE 3.4: Lipids and surfactants used for synthesis of nanoemulsion

3.5.1 Methods of Preparation

The synthesis process was broken into two steps. The first step consisted of preparation of oil and aqueous phase, which was then followed by the emulsification process. The components for the oil phase were carefully measured out into a 50ml beaker (fig 3.5). They were then heated to 45^{o}C to reduce the surface tension and facilitate mixing. 2ml of ethanol was added to facilitate mixing of the oil and extract. This oil phase was then mixed thoroughly under a magnetic stirrer for 3 mins at 1000 rpm. The same procedure was followed for the aqueous phase (table 3.2).

After the oil and aqueous phase had been separately prepared, they were combined under controlled mixing conditions.

To make oil-in-water emulsions, the hot oil phase was added to the aqueous phase drop by drop under various stirring conditions, depending upon the formulation.

The slow, dropwise addition of the oil phase allows the surfactant to efficiently adsorb onto each droplet, stabilizing them and preventing coalescence [101]. The volume of each emulsion was kept at 100ml. The emulsification processes were as follows:

Formulation 1: The hot oil phase was slowly triturated into the aqueous phase under constant magnetic stirring at 1200 rpm for 20 minutes [101].

Formulation 2: The hot oil phase was slowly added into aqueous phase under magnetic stirring at 1200 rpm for 20 minutes. The emulsion then underwent sonication using a bath sonicator at 40Hz for 20 mins. [102].

Formulation 3: The hot oil phase was added slowly to aqueous phase under magnetic stirring at 300 rpm for 5 mins to form a preemulsion. This was then subjected to high sheer mixing using a mechanical stirrer at 12,000 rpm for 15 mins followed by bath sonication for 20 mins [103].

TABLE 3.2: Composition of different nanoemulsion formulations

Formulation	**Oil Phase (% w/v)**	**Surfactant (% w/v)**	**Stabilizer (% w/v)**	**Propolis Extract (% w/v)**	**Nigella sativa Extract (% w/v)**
1	4%	2%	1%	2%	1%
2	3%	2.5%	1%	2%	1%
3	3%	2%	1%	2%	1%

FIGURE 3.5: Nanoemulsion preparation

3.6 Characterization of Nanoemulsion

3.6.1 Particle Size

Nanoemulsion formulations were evaluated for their particle size using Anton Paar 1190 Particle size Analyzer, which uses the principle of laser diffraction. In this technique, a laser beam passes through the sample, and the scattered light patterns are measured. The scattering angle depends on the size of the particles. Larger particles scatter light at smaller angles, whereas smaller particles scatter light at larger angles. Samples were diluted at a ratio of 1:20 with distilled water before analysis to ensure accurate measurements. The instrument provided values for the mean particle size. Data was recorded as mean values by volume, surface area, and number distribution. The formulation with the best particle size and distribution was characterized further and administered to rats [104].

3.6.2 pH

The pH of the emulsion was measured using a digital pH meter. The instrument was first calibrated using three solutions, neutral (pH 7.0), acidic (pH 4.0), and alkaline (pH 10.0). Then pH of the emulsion was measured thrice, without any diluting of the sample and average value was recorded [67]. The pH values of emulsions are influenced by the active drug, the lipid and surfactant used, aqueous conditions and storage conditions. The pH value of emulsions must be ideal for their intended application. Those that are for topical formulations must match the natural pH of the skin to avoid causing irritation, while oral emulsions require a pH compatible with the gastrointestinal tract (2-7) to ensure drug solubility, bioavailability, and stability. Additionally, the pH must not be too low to result in an unpleasant taste and mucosal irritation [105].

3.6.3 FTIR of the Nanoemulsion

Fourier Transform Infrared (FTIR) spectroscopy is an analytical technique that is used to conform used to confirm the chemical composition of a sample and identify functional groups. The FTIR spectra were recorded in the range of 4000-400 cm^{-1}. By comparing the FTIR spectra of the nanoemulsion with those of individual components, any possible interactions such as hydrogen bonds as well as incorporation of ingredients can be identified [67].

3.6.4 Thermal Stability Assessment

The thermal stability of the emulsion was determined by heating a sample to 45^{o}C (fig 3.6) and then cooling another to 4^{o}C. This cycle was repeated 3 times and then the emulsion was observed for any signs of instability, such as phase separation, creaming, or flocculation. This is important for determining how the emulsion behaves under different temperatures, as this can have an impact on its stability during storage and transportation [67, 106].

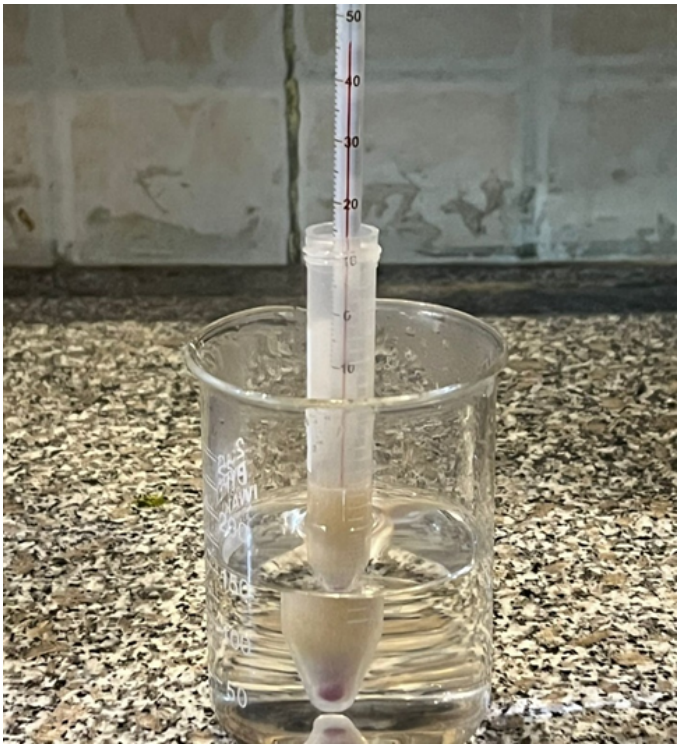

FIGURE 3.6: Heating the nanoemulsion to 45 degrees celsius

3.6.5 Visual Appearance

The emulsion was stored at 25^{o}C and observed for visual changes over a 21-day period. This included changes in color, phase separation, flocculation and creaming. During this period the emulsion was not shaken or disturbed [107, 108].

3.6.6 Dilution Test

Dilution test of the nanoemulsion was performed to confirm nanoemulsion type. For this 1ml of the emulsion was diluted with 10ml of distilled water and shaken well. The mixture was then observed for phase inversion, which can be identified by change from milky to opaque or increase in thickness of the emulsion [109]. Dilution test is a confirmation test for the type of nanoemulsion. It is based on the principle that addition of continuous phase will not disturb the phases. Therefore addition of water, to an oil in water nanoemulsion should not cause phase separation but only dilute the emulsion slightly.

3.6.7 Zeta Potential

Zeta potential is a key parameter in the characterization of nanoemulsions. Zeta potential measures the surface charge of the droplets within the nanoemulsion, which gives a measure of the electrostatic forces of repulsions between the droplets. A higher value, either positive or negative, indicates good repulsion. The higher the charge value, the greater will be the repulsion between the particles will not aggregate together. Ideally a value more negative than -30mV or more positive than +30mV is a good value [109]. Zeta potential can be influenced by the composition of the ingredients as well as storage time and conditions. The zeta potential of the emulsion was measured using Anton Paar litesizer and value was provided.

3.7 Animal Model

In vivo studies were conducted using male *Sprague dawley* rats aged 6-8 weeks, weighing 250-300 g. The rats were housed in separate cages at the CUST animal facility, maintained at a controlled temperature of 22-26^{o}C. The animals were provided with free access to food and water. The rat feed was prepared in 12 kg batches, consisting of 6 kg flour, 1.5 kg choker, 2.5 kg fish powder, and 600 g milk powder. The ingredients were thoroughly mixed and molded into individual feed balls, each weighing approximately 65 g. Clean, absorbent sawdust was used as bedding, replaced daily to maintain hygiene. The sawdust was non-edible, free from contaminants, and provided in sufficient quantities to keep the rats dry and comfortable.

3.8 Induction of Diabetes

To prepare 100 mL of citrate buffer for dissolving streptozotocin, 0.1 M stock solutions of citric acid and sodium citrate dihydrate were made by dissolving 1.05 g of citric acid and 1.48 g of sodium citrate dihydrate in 50 mL of sterile injection water, respectively, and volume was increased to 100ml [110]. The pH was adjusted

to 4.5 (fig 3.7) using sodium hydroxide and hydrochloric acid. Streptozotocin amount was calculated according to the number and weight of the rats and then was dissolved in the buffer immediately before use, with the solution kept on ice and protected from light to preserve stability.

FIGURE 3.7: pH adjustment and preparation of citrate buffer

Rats were placed on food fasting for 8 hours and then administered streptozotocin injections intraperitoneally (fig 3.8) at a concentration of 60mg/kg of body weight [91]. 10% glucose water was immediately administered to rats after giving injection to prevent hypoglycemia and death.

This is important because streptozotocin administration causes the destruction of pancreatic cells leading to release of insulin, which causes initial hypoglycemic state, followed by hyperglycemia. Blood glucose levels were measured 24 hours after injections and successful disease induction was confirmed (fig 3.9) on levels of greater than 200mg/DL [92].

FIGURE 3.8: Streptozotocin injection intraperitonally

FIGURE 3.9: Confirmation of disease induction

3.9 Experimental Design

The study was divided into two parts, toxicity and antidiabetic effects, with separate rats for each part. Rats were divided into groups (Table 3.3 and 3.4) and given dosage via oral gavage for 14 days (fig 3.11). Dosages were calculated by the weight of lipid in the emulsion.

TABLE 3.3: Grouping of rats for evaluation of in-vivo toxicity

Group 1 Negative Control	Received standard diet
Group 2 Low dose nanoemulsion	200mg/kg of body weight [111]
Group 3 High dose nanoemulsion	400mg/kg of body weight [111]

FIGURE 3.10: Grouping of rats for toxicity study

TABLE 3.4: Grouping of rats for antidiabetic evaluation

Group 1 Negative control	Received normal diet
Group 2 Positive control	Disease induction with no intervention.
Group 3 Standard Drug	Metformin 100mg/kg of body weight [112]
Group 4 Nanoemulsion	Nanaoeulsion at 200mg/kg of body weight

FIGURE 3.11: Administration of dosages via oral gavage

3.10 Body Weight and Blood Glucose Measurements

For the 14-day study period, the body weight of each rat was measured regularly using a digital weighing scale. Weights were recorded on day 1, 7 and 14. This data was used to monitor any changes in body weight as an indicator of the rats' health and potential toxicity effects of the nanoemulsion [113]. Blood glucose

measurements for diabetic rats were taken on day 1, 7 and 14 day. To measure blood glucose, the tip of the tail was pricked with a lancet as that portion is very vascular. This caused minimal discomfort to the rat, could be done quickly and allowed enough blood to be released to take a reading.

3.11 Dissection and Blood Collection

On the 15^{th} day of the study, the rats were anesthetized using chloroform. Anesthesia was induced by placing each rat in a chamber containing a cotton pad soaked with chloroform.

Once fully anesthetized, blood was collected via cardiac puncture (fig 3.12). A 23-gauge needle was inserted into the heart at an appropriate angle and blood was gently aspirated [114].

Following blood collection, the animals were carefully dissected using a surgical scissor and No.15 surgical blade. An abdominal incision was made using at the midline of the abdomen (fig 3.12). For toxicity study the livers were removed. For anti-diabetic study the pancreas was removed. Organs were then placed in 10 percent formaldehyde and immediately sent for histopathological evaluation [114].

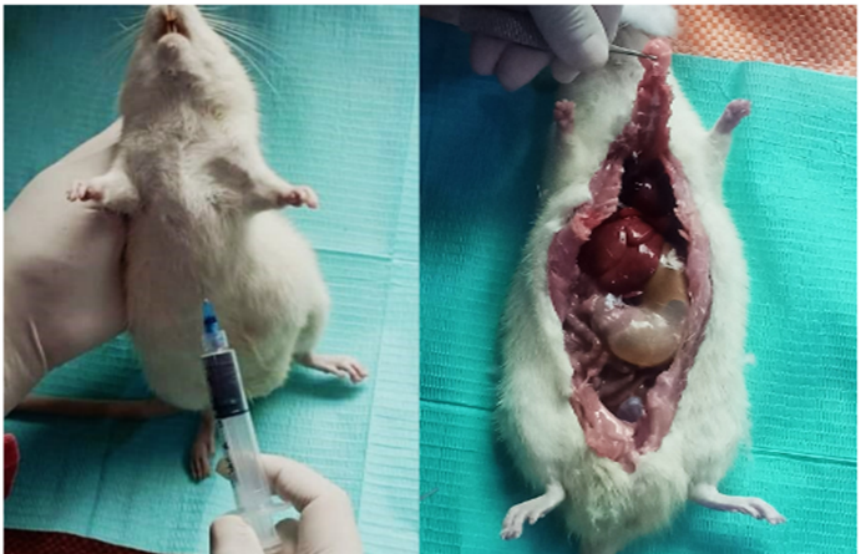

FIGURE 3.12: Cardiac puncture and dissection of rats

3.12 Biochemical Analysis

Blood samples were collected from rats in yellow cap vacuum tubes containing gel and clot activators. For toxicity study, samples were analyzed for liver function tests, including alkaline phosphatase (ALP), alanine transaminase (ALT) and total bilirubin. For antidiabetic study, samples were analyzed for renal function tests including urea and creatinine levels.

3.13 Histopathology

The livers of the toxicity rats and pancreas of the diabetes rats were dissected out and immediately placed in 10% formalin to preserve tissue structure for histopathological analysis (Fig 3.13). Histopathology involves microscopic examination of tissues to study the manifestations of disease. This process helps identify structural cellular abnormalities that may be due to disease or experimental drugs [115].

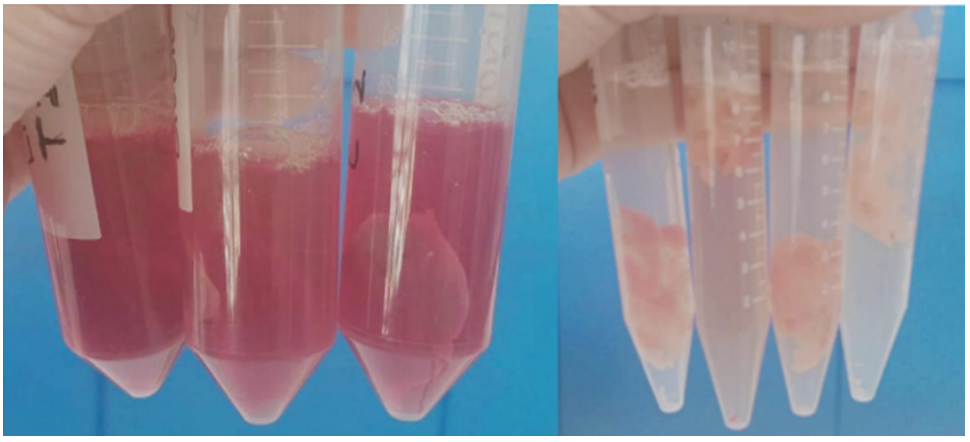

FIGURE 3.13: Liver and pancreatic sample collection for histopathology

3.14 Statistical Analysis

The results in the study were expressed as the Mean ± Standard Error of the Mean (SEM), which provides an estimate of the variability of the sample mean. The SEM helps indicate how precisely the sample mean represents the true population mean.

For statistical analysis, a one-way Analysis of Variance (ANOVA) was used to determine whether there were significant differences among the means of multiple groups. One-way ANOVA is appropriate when comparing multiple independent groups under a single independent variable. After finding a significant difference using ANOVA, Duncan's Multiple Range Test (DMRT) was applied as a post hoc test to identify which specific group means were significantly different from each other [116].

Chapter 4

Results and Discussion

4.1 Extract Preparation

The extraction process of propolis and aimed to achieve maximum yield whilst also preserving the bioactive compounds. Maceration of propolis in 70% ethanol ensured efficient extraction of both polar and non-polar compounds, as ethanol is a versatile solvent for bioactive molecules. Research shows that maximum extraction yield of propolis can be obtained using a 70% ethanol-pure water system [117]. Due to its resinous sticky and waxy nature, propolis must be extracted by soaking in solvents for prolonged periods of time. In the case of ethanol, the optimal maceration time is 10 days and prolonged maceration time of 20-30 days results in a very slight increase in yield of polyphenols [118]. The maceration of *Nigella sativa* seeds within a closed container ensured that volatile compounds such as thymoquinone were not lost by evaporation.

4.2 Fourier Transform Infrared Spectroscopy of Extracts

FTIR is a widely used technique for identifying functional groups and molecular structures based on infrared light absorption. It detects characteristic vibrations of

chemical bonds, producing a unique spectrum for each compound. FITR analysis for extracts of propolis and *Nigella sativa* was done and range was set at 400-4000 cm^{-1}. Baseline correction was then done using Orgin Pro software.

The FTIR spectrum of *Nigella sativa* (fig 4.1) revealed several sharp peaks, each corresponding to specific functional groups that indicate the presence of various bioactive compounds. The peak observed at 3670 cm^{-1} is characteristic of O-H stretching vibrations, which are commonly associated with hydroxyl (-OH) functional groups found in alcohols and phenolic compounds. This suggests the presence of bioactive polyphenols, flavonoids, or other hydroxyl-containing molecules [119]. The peaks at 2927 cm^{-1} and 2850cm^{-1} was assigned to C-H stretching of an aliphatic group, and it implies the presence of methyl and isopropyl components [120].

Another strong C = O stretching band is present at 1719cm^{-1} and denotes the presence of oxygen containing groups such as esters and acids. This peak reflects the presence of thymoquione which shows strong absorption bands and shoulders in the 1700-1600 cm^{-1} [121]. The peak at 1457cm^{-1} is bending vibrations of C-H bonds in methylene groups, reflecting the presence of aliphatic chains in fatty acids [122]. This peak is commonly observed in the FTIR spectra of various oils. Additional peak was noticed at 1177cm^{-1} and is associated with C-O stretching in ester groups of triglycerides [120].

FTIR spectroscopic analysis of propolis (fig 4.2) reveals distinctive functional groups and compounds through its characteristic absorption peaks. The band at 3665cm^{-1} indicates O-H stretching vibrations from phenolic compounds and flavonoids [123]. The distinctive sharp peak at 3242cm^{-1} denotes C-H stretching of aromatics, flavonoids and aromatic rings, whereas the peaks at 2867 refers to C-H elongation of hydrocarbons, indicating the presence of long-chain alkanes, fatty acids, and lipid component. These hydrocarbons may originate from beeswax which is a natural component of propolis. The sharp peak at 1647 denotes bending of C=O of lipids, flavonoids and amino acids. The peak at 1040 represents C-C stretching and C-OH bending of flavonoids and secondary alcohol groups [124].

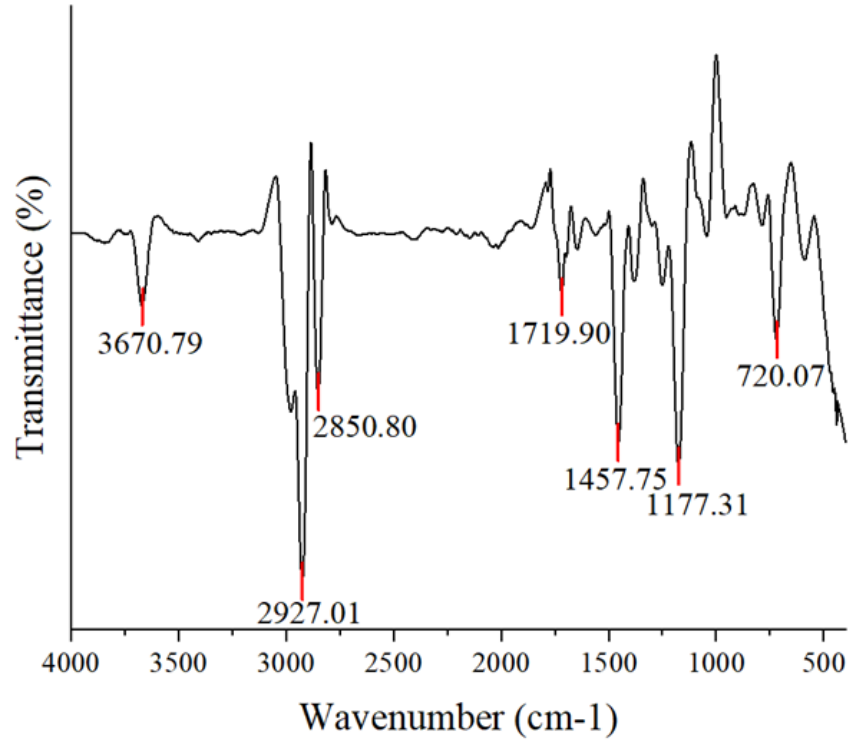

FIGURE 4.1: FTIR of *Nigella sativa* ethanolic extract

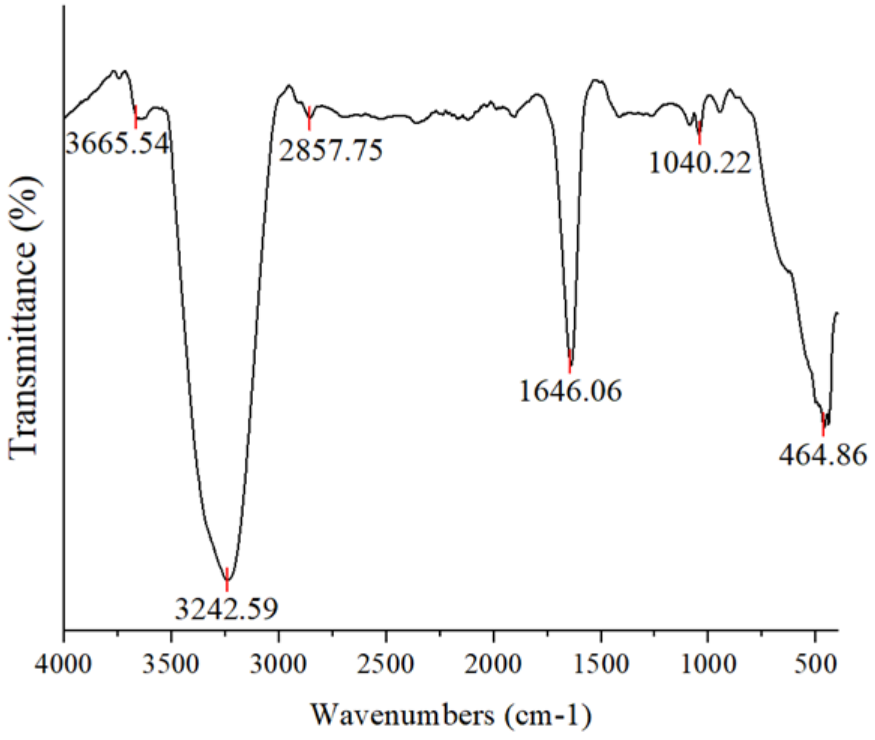

FIGURE 4.2: FTIR of propolis ethanolic extract

4.3 Characterization of Nanoemulsion

4.3.1 Particle Size

Nanoemulsion formulations were evaluated for their particle size using Anton Paar 1190 Particle size Analyzer, which uses the principle of laser diffraction. In this technique, a laser beam passes through the sample, and the scattered light patterns are measured. The scattering angle depends on the size of the particles, with smaller particles scattering light at larger angles and larger particles scattering light at smaller angles [82]. Results were obtained in the form of particle means, by volume, by number and by surface area (Table 4.1), with graphs showing the density and cumulative distribution of the particles.

TABLE 4.1: Volume, number and surface distribution of the nanoemulsions.

Parameter Type	**Formulation 1**	**Formulation 2**	**Formulation 3**
Volume Distribution Mean[nm]	2138.0	435.4	547.0
Number Distribution Mean[nm]	30.67	49.55	30.13
Surface Distribution Mean[nm]	649.0	157.0	245.9

All three formulations showed differences in mean by volume, number and surface area. Formulation 1 showed the largest droplet sizes by volume with a mean of 2138 nm, while showing much smaller sizes when measured by number (30.67 nm) and surface area (649.0 nm). This large difference between volume and number means indicates a highly polydisperse system where a few large droplets dominate the volume distribution while numerous tiny droplets influence the number distribution. Formulation 2 shows more moderate sizes overall, with a volume mean of 435.4 nm, number mean of 49.55 nm, and surface mean of 157.0 nm, suggesting better size uniformity. Similarly, formulation 3 demonstrates intermediate characteristics with a volume mean of 547.0 nm, number mean of 30.13 nm, and surface mean of 245.9 nm. The consistent pattern across all samples where volume means are significantly larger than number occurred as volume measurements are heavily influenced by larger droplets (due to the cubic relationship with radius), while number measurements reflect the predominant population of smaller

droplets. Surface area measurements consistently fall between volume and number values, providing a balanced perspective of the size distribution. The graphical size distribution by volume is represented by two lines. The black line represents the density distribution and indicates the frequency of the particles at each size point. The tip of the bell-shaped peaks shows the most commonly occurring particle sizes.

The cumulative distribution is represented by the blue line and tells the percentage of all the particles in the sample which are smaller than that size. Formulation 1 showed a very irregular distribution (Fig 4.3) with multiple peaks in the density curve, indicating the presence of different size distributions. Formulation 2 showed a single, irregularly shaped curve (Fig 4.4), whereas formulation 3 showed a single well-defined peak (Fig 4.5) with a smooth hyperbolic shaped cumulative distribution curve. Based on this, formulation number 3 was selected and carried further for characterization and administration to rats.

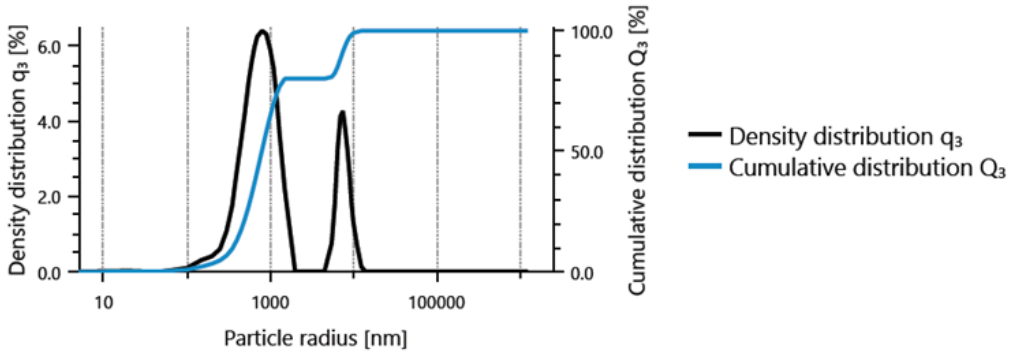

FIGURE 4.3: Volume distribution curve for formulation 1

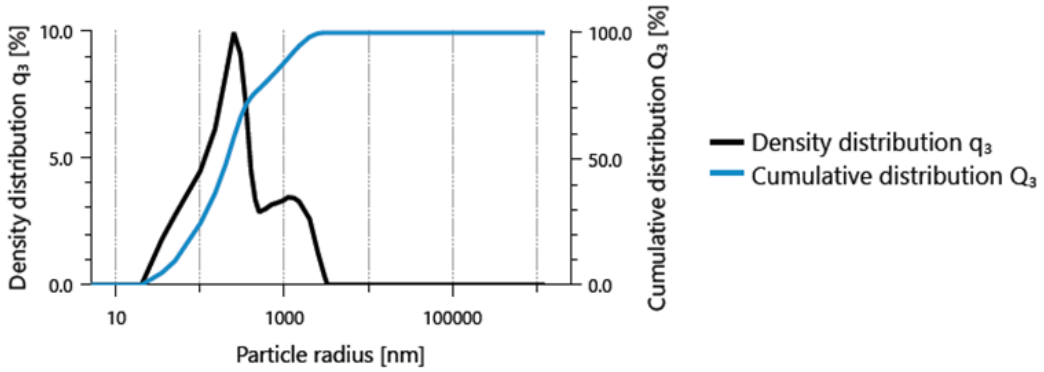

FIGURE 4.4: Volume distribution curve for formulation 2

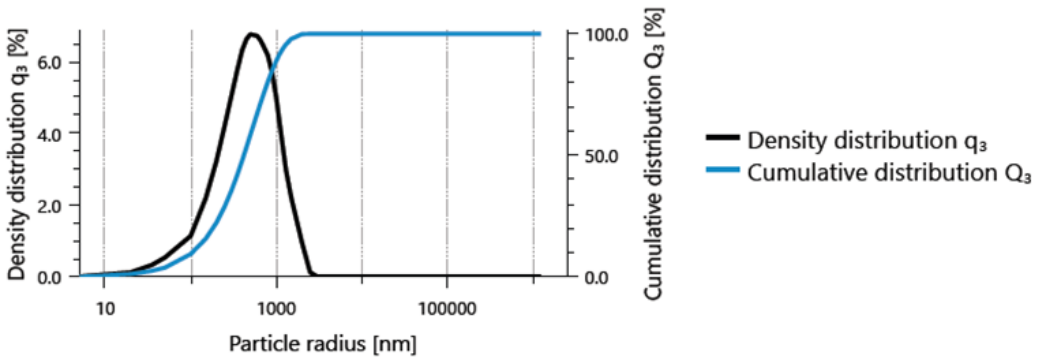

FIGURE 4.5: Volume distribution curve for formulation 3

4.3.2 Fourier Transform Infrared Spectroscopy of Nanoemulsion

FTIR analysis of nanoemulsions is done to confirm functional groups that are present and understand molecular interactions between compounds. It verifies the incorporation of the ingredients and helps understand interactions between the components. When molecules in the nanoemulsion system form multiple hydrogen bonds or other intermolecular interactions, the energy absorption becomes more distributed across different frequencies, leading to broader, flatter peaks instead of sharp ones [125]. This can also indicate incorporation of compounds into the emulsion, formation of new molecular interactions and shielding of functional groups within the emulsion. This is particularly common in O-H stretching regions. FTIR of the nanoemulsion (fig 4.6) showed a broad peak at 3296 and represents merged O-H interactions from water, phenolic compounds from the propolis and hydroxyl groups from *Nigella sativa*. This peak indicates extensive hydrogen bonding between the components. Additionally, the peak present at 1640cm^{-1} denotes successful incorporation of the extracts within the emulsion. Additionally, the peak at 1038 cm^{-1} indicates C-O stretching from flavonoids in propolis and surfactant C-O bonds. These peaks indicate successful encapsulation of the extracts into the emulsion [126].

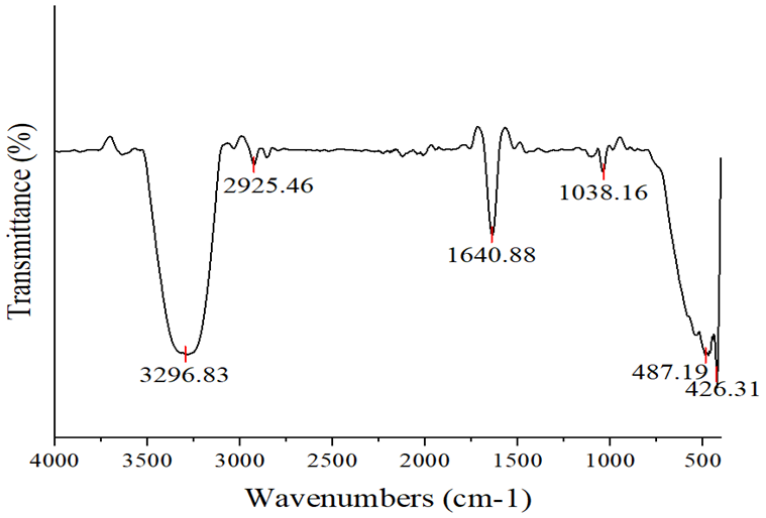

FIGURE 4.6: FTIR of nanoemulsion of Propolis and *Nigella Sativa*

4.3.3 pH

The pH of the nanoemulsion was determined using a digital pH that had been previously calibrated. Three readings were taken, and mean value came out to be 4.8. The slightly acidic nature of the emulsion can be attributed to its components. Oleic acid is a weak acid, and has a pka of approximately 5.0, meaning it only partially dissociates in water [127]. Tween 80 on the other hand, when dissolved in water shows a pH of 6 [128]. Propolis has a slightly acidic pH, with a study reporting values between 4.2-5.2 [129]. Emulsions given orally must have a pH of between 5 to 7, aligning with the normal pH of the oral cavity. This ensures good compatibility and less irritation when administered orally [130]. Hence the prepared emulsion was close to the optimal pH for oral suspensions.

4.3.4 Thermal Stability

Thermal stability of the emulsion was checked by subjecting it to various temperature changes. The emulsion was cooled to 4 degrees and heated to 45 degrees for 3 cycles and then visually observed for any phase separation, or creaming. No phase separation or any visual signs of instability were noticed, indicating that the emulsion was stable at such high and low temperatures. Nanoemulsions by nature

are kinetically stable, but thermodynamically unstable. This means that when the temperature increases, the kinetic energy of the droplets increases, leading to increases movement and collisions with other droplets, which can lead to coalescence of the droplets and phase separation [130]. Nanoemulsions, especially those intended for pharmaceutical use, must be able to withstand multiple temperature fluctuations during storage and still retain their properties [131].

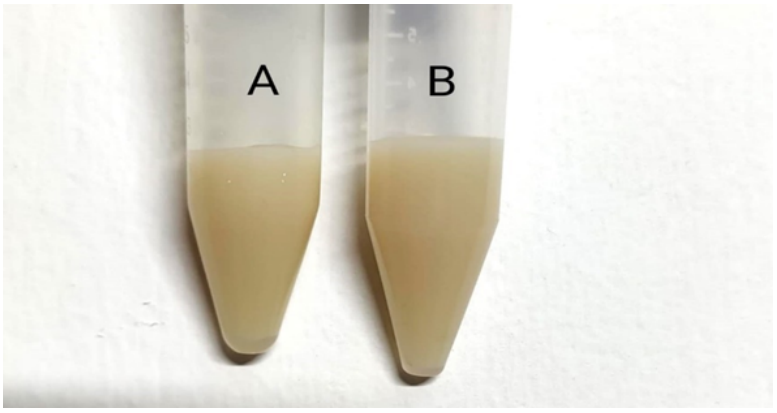

FIGURE 4.7: (A) emulsion at room temperature (B) emulsion after 3 cycles of cooling and heating

4.3.5 Visual Stability

The emulsion was stored at room temperature for a period of 21 days (Fig 4.8) in a centrifuge tube. During this time the emulsion was not shaken or moved and was inspected every 7 days for any visual changes such as phase separation, creaming or flocculation [132] During the course of the 21 days the emulsion did not show any sign of phase separation at the macroscopic levels. This can be attributed to the small droplet size and compatibility of the lipid and surfactant phase. These findings are consistent with other studies that use the same lipid and surfactant. A study investigating the anti-fungal activity of a microemulsifying delivery system using oleic acid and tween 80 showed good stability [133]. Another research formulated nanoemulsions using oleic acid and tween 80 and showed stability for up to 12 months when stored in the dark, indicating compatibility between oleic acid and tween 80 [134].

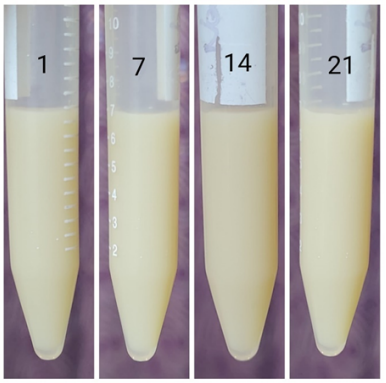

FIGURE 4.8: Visual appearance of emulsion over 21 days

4.3.6 Dilution Test

Dilution test was performed on the oil-in-water nanoemulsion. 1 ml of the emulsion was taken in two separate test tubes. 10 ml of distilled water was added to the first tube with the same amount of oleic acid to second tube. Both tubes were gently shaken and evaluated after 5 minutes. Dilution test is a simple test that allows confirmation of the type of nanoemulsion. It is based on the principle that addition of more continuous phase will only dilute the emulsion and not destabilize it [109]. Addition of water confirms that the emulsion was oil-in-water type as no phase separation or precipitation occurred. When oleic acid was added, the emulsion did not mix, and two distinct layers of oleic acid and emulsion were seen (Fig. 4.9)

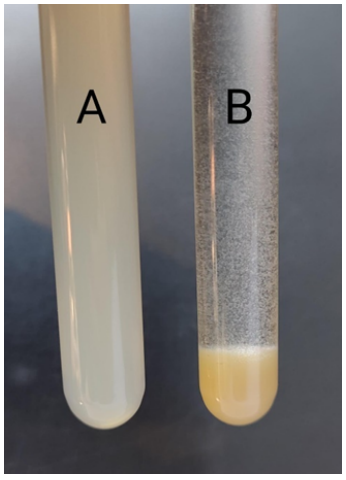

FIGURE 4.9: (A) Emulsion after addition of distilled water, (B) emulsion after addition of oleic acid

4.3.7 Zeta Potential

The zeta potential of the nanoemulsion was measured using Anton Paar litesizer. Zeta potential is a measurement of the surface charge of droplets within the emulsion. A high value of zeta potential, either positive or negative is desirable as the stronger the charge on the surface of the droplets the better the repulsion between the particles. Ideally a value more negative than -30mV or more positive than +30mV is a good value [109]. The zeta potential of the nanoemulsion was -23, However these findings are consistent with another study that developed beeswax oleic acid emulsions using Tween 80 as a surfactant [135].

4.4 Safety Profile

To evaluate toxicity effects of the nanoemulsion rats were divided into three groups. Group one was a normal control group which received a normal diet for the duration of the study. The second group received a low dosage of the nanoemulsion and the third group received high dosage of the nanoemulsion. Body weights were monitored through the duration of the study. On the 14th day the rats were euthanised and blood samples were taken for liver function tests. The rats were dissected, and livers were sent for histopathological evaluation.

4.4.1 Body Weight

Body weight was measured on the 1st, 7th and the 14th day of the study. Results show that despite oral administration of nanoemulsion, there was no significant change in mean body weights between groups (table 4.2) . This could indicate that despite giving the emulsion orally, it did not have an overall systemic effect on the body. Many substances and drugs, including lipids and surfactants may cause localized effects and induce mild biochemical changes, without causing a significant overall toxic effect on the body.

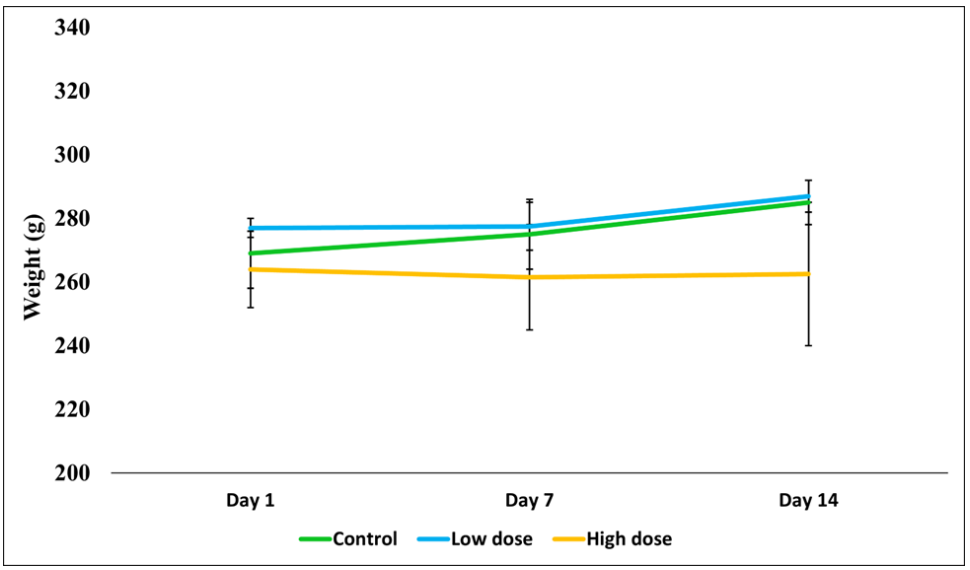

FIGURE 4.10: Weights of experimental groups in toxicity study

TABLE 4.2: Body weights, one-way Anova between the experimental groups along with mean, ± standard error, Duncan Multiple Range Test.

	Day 1	**Day 7**	**Day 14**	**p-value**
Control	269±11a	275±11a	285 ±7a	0.573
Low-Dose	277±3a	277.5±7.5a	287±5a	0.450
High-Dose	264±12a	261.5±16.5a	262.5±22.5a	0.9

4.4.2 Biochemical Assays

Liver function tests, including alkaline phosphatase (ALP), alanine aminotransferase (ALT), and bilirubin levels, were conducted to assess the potential hepatotoxic effects of the lipid nanoemulsion in rats. Elevated levels of these biomarkers indicate liver damage or dysfunction, while normal values suggest the nanoemulsion's safety. These parameters were chosen due to their sensitivity in detecting hepatic injury, cholestasis, and overall liver health.

4.4.2.1 Alkaline Phosphatase

Alkaline phosphatase is an enzyme that is found throughout the body, but is primarily concentrated within the liver, within the bile duct cells. It is used

as a biomarker in toxicity studies because it provides information regarding liver damage. ALP levels may increase when hepatotoxic substances affect the liver, and cause inflammation and damage to bile duct cells [136]. The results show that there was a significantly increased level of ALP within the group of rats administered a high dosage of the nanoemulsion (table 4.3). This can be attributed to the presence of Tween 80 or polysorbate 80 that was present within the emulsions. Tween 80 is used as a surfactant within food, pharmaceutical and cosmetics, however due to concerns with toxicity, the FDA has strictly limited its use of no more than 1% within food products [137]. Research shows that increased levels of the surfactant within both food and water can cause an increase of ALP levels, as the molecule interacts with bile duct cell membranes triggering a response [138]. Furthermore, the results align with studies that show that orally administered polysorbate 80 can influence the gut microbiota of both mice and rats leading to inflammation and changes in ALP levels [139].

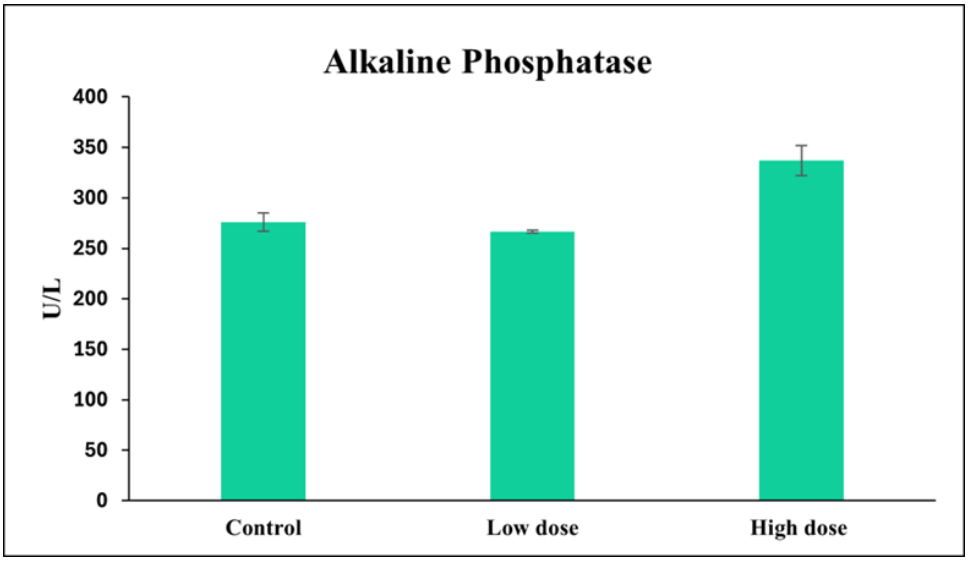

FIGURE 4.11: Alkaline Phosphatase levels of experimental groups

TABLE 4.3: ALP, one-way Anova between the experimental groups along with mean, ± standard error, Duncan Multiple Range Test.

Parameter	Control	Low-Dose	High-Dose	p-value
ALP	276±9a	266.5±1.5a	337±15b	0.029

4.4.2.2 Alanine Aminotransferase

Alanine aminotransferase is an enzyme that is predominantly present within the liver and is used as a marker in hepatocellular injury. It catalyzes the chemical reaction that transfers amino groups between the amino acids alanine and glutamate. Whenever the liver is directly damaged, cells release the enzyme leading to rise in serum levels. The results show that the emulsion did not cause a significant increase in the ALT levels between the experimental groups as the p value was greater than 0.05 (table 4.4). These findings align with previous research that show that oleic acid can induce fat accumulation (steatosis) in liver cells, but it does not necessarily lead to hepatocellular injury or liver enzyme elevation [140]. Interestingly this could indicate that the emulsion, which caused increased in ALP levels without significant increase in ALT levels, may have primarily exerted its effect on the bile duct cells.

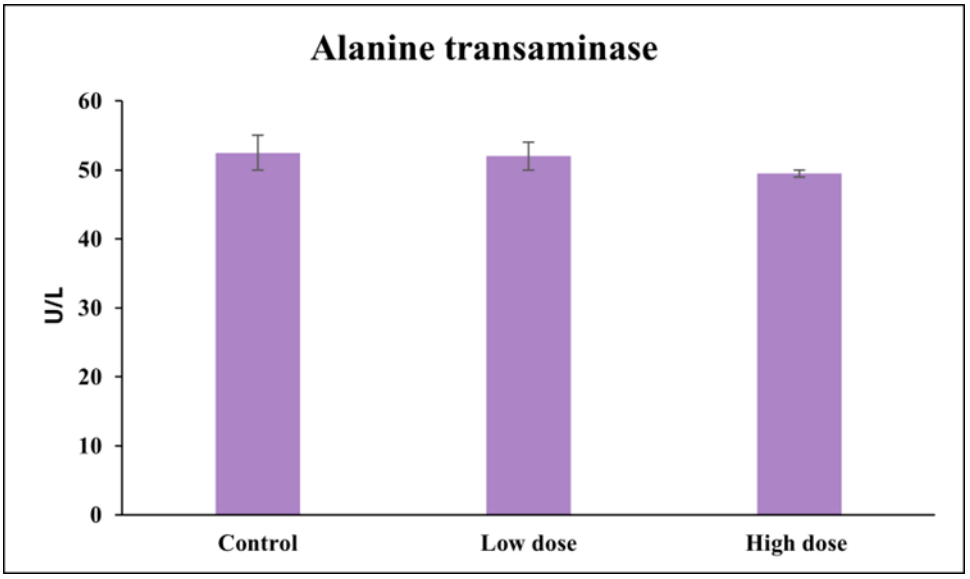

FIGURE 4.12: Alanine transaminase levels of experimental groups

TABLE 4.4: ALT, one-way Anova between the experimental groups along with mean, ± standard error, Duncan Multiple Range Test.

Parameter	Control	Low-Dose	High-Dose	p-value
ALT	52.5±2.5a	52±2a	49.5±.5a	0.549

4.4.2.3 Bilirubin Total

Bilirubin is a yellow-colored pigment that is a byproduct of heme and is produced when the red blood cells within the body are broken down. This bilirubin then travels to the liver where it is processed and then excreted into the bile. Levels within blood may increase either due to excessive red blood cell destruction, or if there is significant damage to the liver in which case it is not processed correctly. Levels may also rise when there is damage to the bile duct [141]. The results of the study indicate that the bilirubin levels did not change significantly between the experimental groups as the p value was greater than 0.05 (table 4.5).

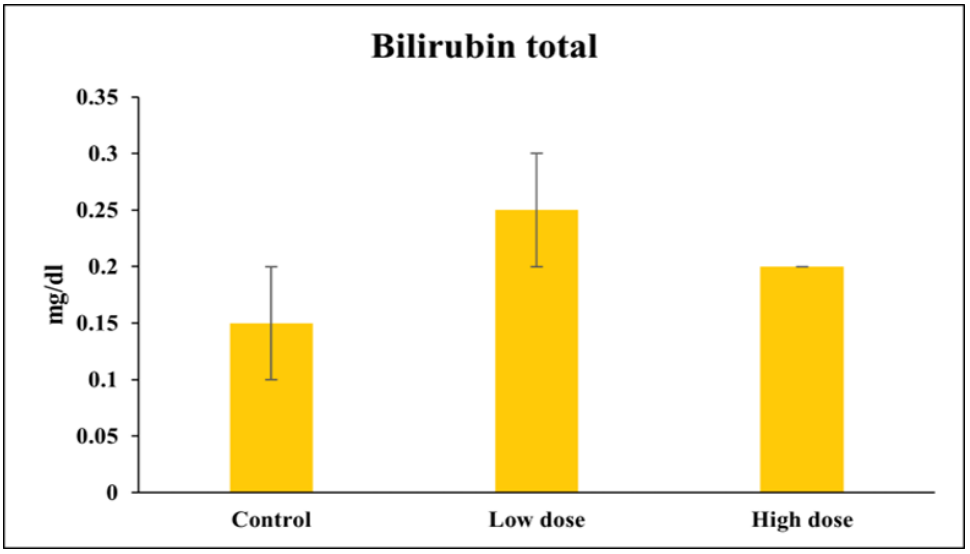

FIGURE 4.13: Bilirubin total levels of experimental groups

TABLE 4.5: Bilirubin, one-way Anova between the experimental groups along with mean, ± standard error, Duncan Multiple Range Test.

Parameter	Control	Low-Dose	High-Dose	p-value
Bilirubin Total	0.15±0.05a	0.25±0.05a	0.2±0a	0.35

4.4.3 Histopathology

On the 14^{th} day the rats were euthanized and livers from normal, low dose and high dose group were dissected out and placed in 10% formalin and sent for histopathological evaluation. The liver of the negative control group appeared normal, there

were no signs of necrosis or inflammation. The histopathology of low dose group also indicated minimal change and appeared normal, consistent with findings of biochemical tests. The high dose group showed very early signs of mild inflammation in localized regions, but otherwise the tissue architecture remained the same.

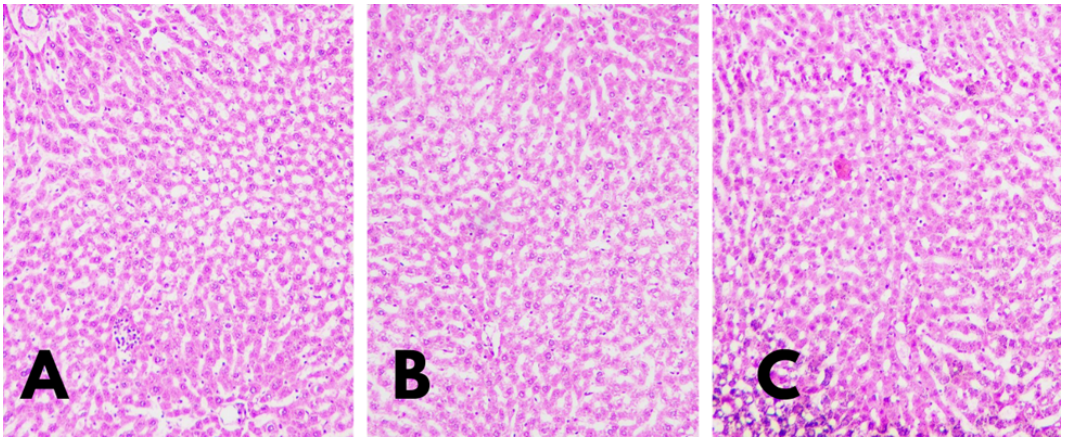

FIGURE 4.14: Histo analysis of (A) Negative control, (B) Low dose and (C) High dose nanoemulsion

4.5 Antidiabetic Evaluation

To determine the anti-diabetic effects of the nanoemulsion, rats were divided into four groups. The negative control group was non-diabetic and received normal diet and water over the course of the study. The positive control group was diabetic rats; however, they received no drug or intervention. The third group was a standard drug group, which received metformin at a dosage of 100mg/kg of body weight daily. Lastly the nanoemulsion group received the emulsion at a dosage of 200mg/kg of body weight.

Body weights were taken on the 1^{st}, 7^{th} and 14^{th} day of the study along with fasting blood glucose levels. On the 15^{th} day, the rats were euthanised and blood samples were collected for biochemical evaluation, specifically renal function tests. The pancreas of all groups was dissected out and sent for histopathological evaluation.

4.5.1 Body Weight

After induction of diabetes, the body weights of the rats among the diabetic groups showed extreme fluctuations in weight as shown in table 4.6. The negative control group did not undergo any significant variation over the experimental period. This indicates that the rats were in good health and maintained normal growth over the duration of the study.

TABLE 4.6: Body weights (Diabetic groups), one-way Anova between the experimental groups along with mean, ± standard error, Duncan Multiple Range test.

	Day 1	**Day 7**	**Day 14**	**p-value**
Control	269±11a	275±11a	285 ±7a	0.573
Positive Control	343±8.5b	311±9b	277±2a	0.017
Standard Dose	357.5±2.5b	337±2a	335±5a	0.033
Emulsion	332.5±4.5b	300.5±4.5a	295±0.0a	0.010

On the other hand, the positive control group underwent drastic weight loss, with almost 20 percent reduction in body weight over the course of the experiment. This dramatic decrease in weight is consistent with the findings of diabetic studies, where high dosages of streptozotocin can lead to weight reduction in the days following induction [143]. Streptozotocin, due to being a glucose analog, destroys the beta cells of the pancreas, leading to a deficiency in insulin and subsequent hyperglycemia. This increases the urine output to rid the body of the extra glucose, through osmotic diuresis, which can lead to dehydration in severe cases and contribute to weight loss [144, 145].

Additionally, hyperglycemia along with insulin deficiency and resistance, means that the body tissues can no longer take up glucose from the blood and use it for energy. This causes breakdown of fat and muscle in a process called lipolysis and proteolysis, which further contributes to weight loss [146].

The group that received the standard drug metformin showed a moderate reduction in weight, which was less drastic compared to the positive control. This suggests that the drug had some beneficial antidiabetic effects, likely improving insulin sensitivity and glycemic control.

Metformin does this by acting on the liver inhibiting hepatic gluconeogenesis, a process by which the liver produces glucose, thus improving the insulin sensitivity within the peripheral tissues. This leads to less breakdown fat for glucose production [147].

These findings suggest that metformin was able to counteract some of the metabolic dysfunctions that arose due to streptozotocin within the positive control group.

The emulsion group, which received the nanoemulsion containing propolis and showed a notable reduction in weight loss. This suggests the emulsion showed some positive effects on metabolism compared to the positive control group as the weight loss was not as severe as the positive control group.

Propolis is a waxy resinous substance obtained from bee hives and is rich in phytochemicals that have beneficial pharmacological effects. Research shows that propolis can improve insulin sensitivity in diabetic patients [148].

This could lead to more utilization and uptake of glucose from the blood and less breakdown of fat for energy production. *Nigella sativa*, also present within the emulsion has been demonstrated to improve blood glucose and insulin levels, mitigate the complications of diabetes and stimulate glucose absorption [149].

These findings also suggest that the nanemulsion that was stabilized by oleic acid and tween 80, was able to produce a stable emulsion that was effectively absorbed into the bloodstream from the gastrointestinal tract.

Oleic acid, a fatty acid that is a component of peanut and olive oil, could contribute to the weight maintenance of the rats. Research shows that oleic acid itself has antidiabetic effects, as it protects against insulin resistance by regulating genes involved in the PI3K pathway [150].

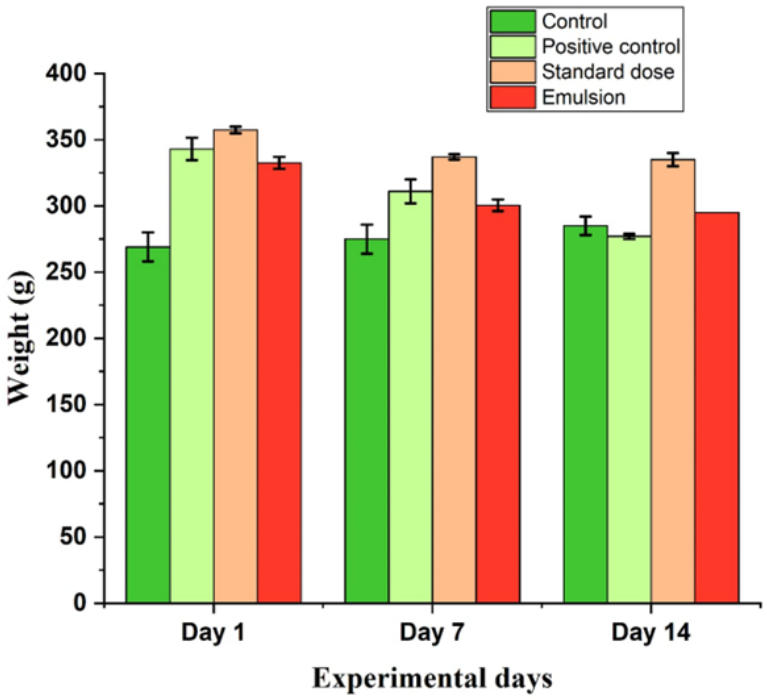

FIGURE 4.15: Body weights of diabetic experimental groups

4.5.2 Blood Glucose Levels

The fasting blood glucose levels across all experimental groups displayed significant differences and variations (table 4.7). The negative control groups remained stable with regards to weight as well as blood glucose levels. This can be attributed to the presence of a normally functioning pancreas with insulin production to maintain glycemic control. The rats did not undergo any significant weight loss or gain, indicating that their metabolism was relatively stable throughout the duration of the study. In contrast the positive control group displayed increasing levels of blood glucose, indicating sustained and increasing hyperglycemia. This can be correlated with their weight loss as lack of insulin, increasing levels of glucose in the blood and inability for tissues to use the glucose lead to fat breakdown for energy.

Within the standard drug group, there was a significant reduction in blood glucose by the end of the 14th day, with only 8 percent body weight loss compared

to almost 20 percent in the positive control group. This indicates that the metformin had a positive effect on glycemic control, and despite the severity of disease induction was still able to exert some anti-diabetic effects. Metformin primarily reduces hepatic gluconeogenesis and increases peripheral glucose uptake, leading to reduced blood glucose levels and weight modulation.

The nanoemulsion group showed also showed reduction in blood glucose levels which was not as pronounced as the standard drug group but were still significant according to p values. This indicates that the emulsion was stable, was able to encapsulate the active drug and be absorbed into the blood stream from where it reached the peripheral tissues and exerted its effect.

This is demonstrated by comparison with the positive control group, which not only showed a severe drop in weight but also increasing hyperglycemia. The emulsion, in contrast, was able to reduce the blood glucose levels to a small extent whilst also preventing them from increasing, indicating a therapeutic effect through successful delivery of the active ingredients to the body cells. It is possible that further reduction in blood glucose levels could have been attained if a higher dosage was used.

The therapeutic effect of the emulsion can be attributed to the synergistic action of its ingredients. Studies show that replacing unhealthy fats such as saturated fatty acids, with a healthier option such as oleic acid can help improve insulin sensitivity. A study conducted on pancreatic beta cells lines showed that oleic acid even has the capacity to stimulate release of insulin from the beta cells [151]. This can explain why people with Mediterranean diets rich in peanut and olive oil have better glycemic controls and lesser risk of developing insulin resistance [152]. Both propolis and *Nigella sativa* have well established anti-diabetic effects and have been tested in both humans and animals, however like most drugs they also face issues with poor solubility and bioavailability [153, 154]. Hence the results show that the extracts were successfully encapsulated and delivered to the body cells.

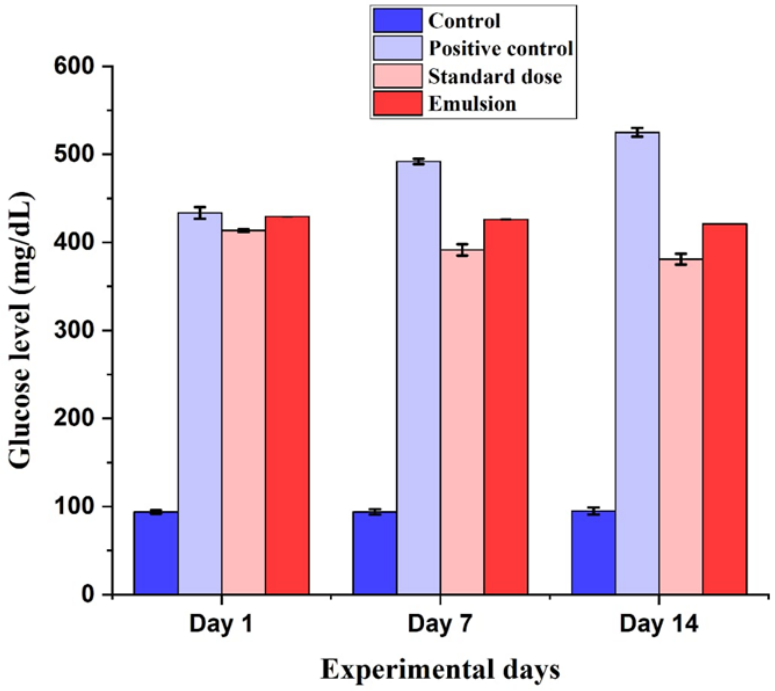

FIGURE 4.16: Blood glucose levels of diabetic experimental groups

TABLE 4.7: Blood glucose levels, one way Anova between the experimental groups along with mean, ± standard error, Duncan Multiple Range Test.

	Day 1	**Day 7**	**Day 14**	**p-value**
Control	94±2a	94±3a	95 ±4a	0.96
Positive Control	433.5±6.5a	492±3b	525±5c	0.002
Standard Dose	413.5±1.5b	391.5±6.5ab	381±6a	0.046
Emulsion	429.5±0.5c	426±1b	421±0.5a	0.009

4.5.3 Biochemical Assays

Blood samples from diabetic group rats were collected and evaluated for renal function tests, including urea and creatinine levels, in order to comprehensively determine the antidiabetic effects of the emulsion.

4.5.3.1 Urea and Creatinine

Urea and creatinine are waste products that the kidney filters from the blood, and their levels are important indicators of renal function. Urea is produced when the

liver breaks down proteins and the waste is excreted out by the kidneys. High levels of urea may indicate kidney dysfunction, dehydration, or excessive protein intake, while low levels may indicate liver disease or malnutrition. Creatinine is a byproduct of muscle metabolism, and its levels are relatively constant in individuals with stable muscle mass. It is filtered out by the kidneys, and an elevated creatinine level can suggest kidney impairment. Creatinine levels are often used to estimate the glomerular filtration rate (GFR), which is a measure of kidney function [155].

Within antidiabetic study's renal function tests are frequently conducted. This is because persistently high blood glucose levels can lead to renal damage, as all the glucose must be filtered out from the blood into the urine. This leads to a condition called diabetic nephropathy, that is characterized by elevated levels of both urea and creatinine [156].

The results indicate that there were statistically significant differences in urea between the experimental groups (table 4.8). The positive control group had an elevated urea level compared to the negative control groups. This can be attributed to to reasons. Firstly, this group showed blood glucose levels reaching 500mg/dl by the end of the study, indicating severe hyperglycemia. An additional observation noted during the study was that all diabetic groups, especially the positive control groups, increased their urine output, due to frequent refilling of water compared to negative control group.

This indicates that the excess amount of glucose within the blood was being filtered out rapidly by the kidneys, leading to renal impairment and glomerular damage. Secondly, all rats received a high dosage of streptozotocin, of 60mg/kg of body weight. Research shows that streptozotocin can lead to renal impairment through direct and indirect means [157]. Indirectly, the chemical damages the beta cells of the pancreas, leading to insulin deficiency and hyperglycemia, which contributes to diabetic nephropathy. However, the chemical can also directly cause damage to the proximal tubules within the kidneys [158].

The results show that both urea and creatinine levels were decreased in the standard drug group and the emulsion compared to the positive control group. However, according to statistical analysis the differences in creatinine were not statistically significant. The group receiving the emulsion, however, showed better reduction that the standard drug. This can be attributed to the therapeutic effects of the extracts. Propolis exerts a protective effect on the integrity of the renal tissue membrane.

Treatment of CCl4-damaged murine renal tissue with propolis led to better kidney structure and less glomerulus swelling compared to the untreated tissue [159]. Another study showed that treatment of diabetic rats with propolis significantly decreased both creatinine and urea levels [160]. Hence the findings of the study are consistent with published literature. Additionally, propolis also has shown to have anti-inflammatory effects, by decreasing expression of inflammatory genes and reducing influx of immune cells, all of which contribute to improvement of renal function [161].

Furthermore, a study investigating the effects of *Nigella sativa* on renal function in rats also showed significant reduction in creatinine levels compared to control rats [162].

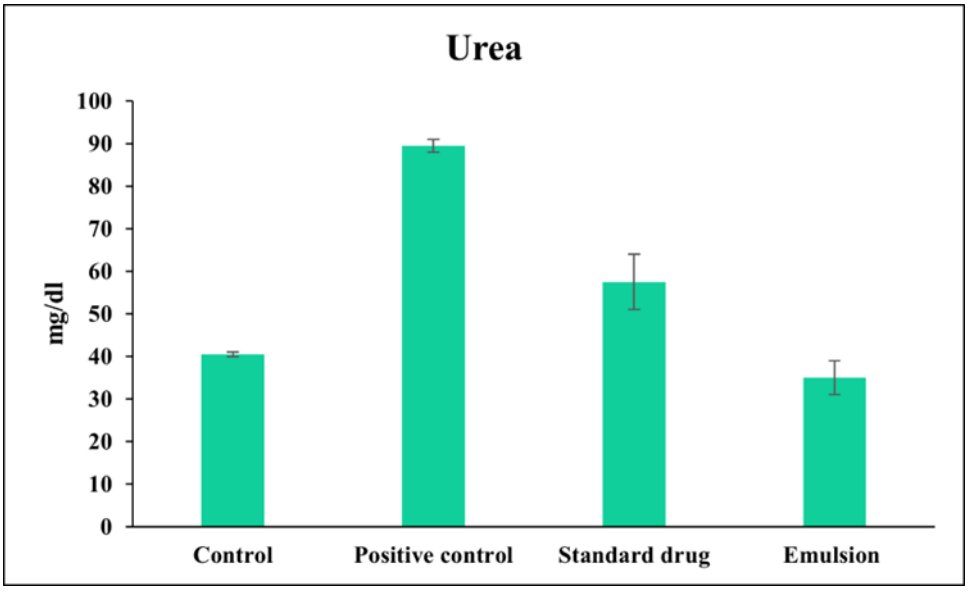

FIGURE 4.17: Urea levels of experimental groups

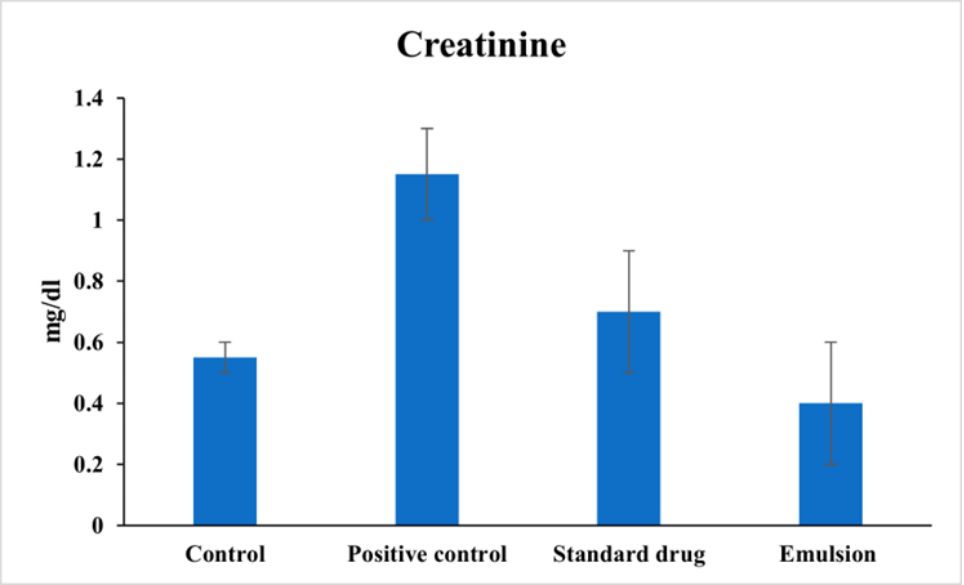

FIGURE 4.18: Creatinine levels of experimental groups

TABLE 4.8: Renal function tests: one way Anova between the experimental groups along with mean, ± standard error, Duncan test.

Parameter	**Control**	**Positive control**	**Standard Dose**	**Emulsion**	**p-value**
Urea	40.5±0.5a	89.5±1.5c	57.5±6.5b	35±4a	0.002
Serum Creatinine	0.55±0.05ab	1.15±0.15a	0.7±0.2a	0.4±0.2a	0.107

4.5.4 Histopathology

On the 15^{th} day of the study, the rats were euthanized, and pancreas were dissected. Tissue samples were placed in 10 percent formalin and sent for histopathological evaluation. The morphology of the negative control group was normal. There were no indications of inflammation or structural anomalies, and the tissue's acini, islets of Langerhans, and ducts all seemed to be in good condition. There were no pathological alterations seen since the cells were well-organized and displayed normal shape and function. The positive control group, on the other hand, displayed noticeable destruction of pancreatic tissue architecture, with visible degeneration and cellular injury with inflammatory cell infiltration. Both the standard drug and emulsion groups displayed prominent histopathological changes,

with areas of degeneration, destruction of tissue architecture and inflammatory cell infiltration.

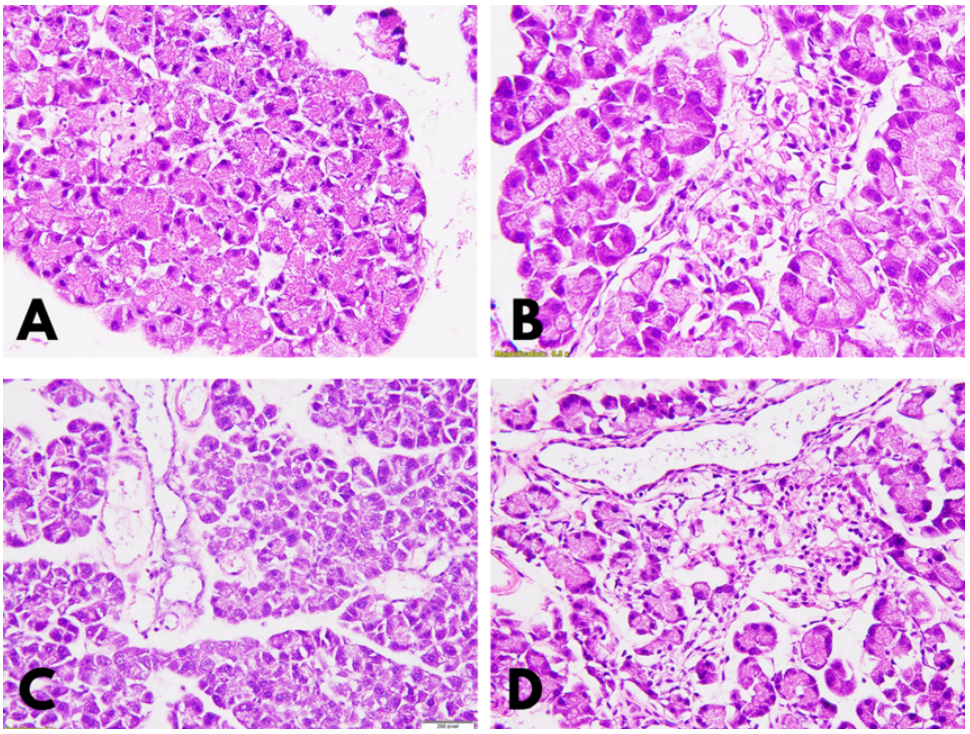

FIGURE 4.19: Histo analysis of (A) Negative control, (B) Positive control, (C) Standard drug and (D) Nanoemulsion

Chapter 5

Conclusion and Future Directions

This study utilized oleic acid, Tween 80, and soy lecithin as the lipid and surfactant components to formulate and characterize a lipid nanoemulsion incorporating propolis and *Nigella sativa* extracts. The emulsion demonstrated favorable physicochemical properties, including optimal particle size, stable pH, and as well as thermal and long-term stability. Toxicological assessments revealed that the formulation did not significantly impact alanine aminotransferase (ALT), bilirubin levels, or body weight, suggesting a good safety profile at lower dosages. However, a slight increase in alkaline phosphatase (ALP) levels was observed at higher doses which could be attributed to the presence of surfactants.

In the antidiabetic evaluation, the nanoemulsion-treated group exhibited improved renal function parameters and superior glycemic control compared to the untreated diabetic controls. This suggests that the synergistic effects of propolis and *Nigella sativa* in the nanoemulsion system hold promise for managing diabetes and its associated complications. The observed therapeutic benefits may be attributed to the bioactive compounds within these natural extracts, which have established antioxidant, anti-inflammatory, and hypoglycemic properties.

Given the rising global and national prevalence of diabetes, particularly in Pakistan, there is an urgent need for accessible and cost-effective therapeutic interventions. This study highlights the potential of using lipids, surfactants, and bioactive extracts from propolis and *Nigella sativa* as key formulation components that are

not only readily available but also cost-effective and therapeutically beneficial. Developing such an emulsion as an alternative to conventional synthetic medications could provide a safer and more affordable treatment option for diabetic patients.

Future research should focus on optimizing the nanoemulsion formulation to enhance its efficacy, stability, and safety profile. This includes investigating the impact of different surfactant combinations to minimize any potential adverse effects while ensuring long-term stability. Additionally, higher dosage studies in animal models should be conducted to assess long-term efficacy. Further exploration into the pharmacokinetics and bioavailability of the active compounds within the nanoemulsion is necessary to determine optimal dosing strategies.

Future research should also focus on determining its effectiveness in combination with other therapies, as well as evaluating its long-term stability under different temperature and storage conditions. Addressing these factors could contribute to the development of a natural, effective, and widely accessible therapeutic option for managing chronic diseases.

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