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Potential prophylactic effect of Naringenin loaded liposome eye drops on streptozotocin-induced diabetic cataract in rats

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Abstract

Diabetes may lead to oxidative stress-related ocular complications including cataract. Thus, the aid of antioxidant therapy versus oxidative stress development is necessary to reduce cataract progression. Flavonoid Naringenin is a well- recognized natural antioxidant. Liposomes are appropriate nano-carriers for ocular drug delivery. Hence, we aimed to prepare and assess the potential of Naringenin loaded liposome (NLL) as topical ocular drug to ameliorate streptozotocin (STZ)-induced diabetic cataract. NLL was formulated by thin lipid film method (0.241 mg/ml); its characterization was confirmed by Transmission Electron Microscope (TEM) imaging, zeta potential and Differential Scanning Calorimetry **(**DSC) measurements. 60 male Wistar rats have been distributed into three groups (control, (STZ) - induced diabetic cataract and Naringenin treated). NLL was administered as eye drops (in treated group) twice a day for 12 weeks. Slit-lamp photography and lens inspection were conducted, FBS was detected. In the lens tissue reduced glutathione (GSH), total soluble lens protein (TSP), carbonyl protein (CP), interleukin- 6 (IL-6), Caspase-3 and vascular endothelial growth factor (VEGF) were measured. TEM imaging, particle size, zeta potential and DSC results exhibited an effective NLL preparation. NLL exhibited antioxidant, anti-inflammatory and antiapoptotic efficacy in all parameters associated with oxidative stress in the lens tissue of type 1 diabetic rats. Finally, the findings disclosed that NLL could be recommended as a potential prophylactic agent in diabetic cataract.

Keywords: Naringenin; Liposomes; DSC; Diabetes; Cataract; Oxidative stress.

1. Introduction

Diabetes mellitus (DM) be present a persistent metabolic disease marked by hyperglycemia brought on by inadequate insulin production and/or impaired insulin action. Diabetes comes in numerous types, with type 1 and type 2 being the most prevalent [1]. Globally, 8.4 million people had diabetes (type 1) in 2021; of these, 1.5 million (18%) were under 20 years old, 5.4 million (64%) were between 20 and 59 years old, and 1.6 million were older than 60 years [2].

In T1DM, a number of harms are typically done to β-cells, which ultimately results in β-cell death. Due to the low activity of antioxidant enzymes, islet β-cells are vulnerable to damage from reactive oxygen species (ROS), which can directly damage islet β-cells and activate numerous redox-sensitive signaling cascades, resulting in mitochondrial damage [3]. Uncontrolled hyperglycemia in diabetics can cause ocular diabetic complications, in which the diabetes can change the metabolism of the lens, resulting in cataracts and blindness [4]. Cataract affects 95 million people globally, according to estimates. The primary cause of blindness in middle-income and low-income nations continues to be cataract [5], the major recognized pathogenesis of diabetic cataract is polyol pathway, oxidative stress pathway and glycosylation pathway [6-8].

Under diabetic conditions, the control of blood glucose is necessary to reduce cataract progression and severity [9]. This cannot be achieved all the time, so the aid of supplementary therapy interfering with the progression of the disease, relies on antioxidants and anti-glycation medicines is needed to slow the progression of the disease and lessen the harmful impact of diabetes on lenses. Numerous pathways are involved in the opacification of the eye lens, including polyol pathway, non-enzymatic glycation and oxidative stress; Flavonoids are being investigated as probable medicines that may reduce the formation of cataract [9].

Naringenin is a polyphenol belonging to flavonoids; is primarily present in citrus fruits like grapefruit, orange juice, and lemon juice [10]. This compound exhibits antihyperglycemic properties; it is also a promising treatment for diabetic complications brought on by oxidative stress [11, 12]. Many studies have shown

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that Naringenin, when administered topically, orally, or intraperitoneally in experimental models produces a favorable ophthalmological effect [13, 14]. When medications are applied topically to the eye surface, they must cross the corneal barrier to reach the interior segments of the eye; the hydrophilic stroma restricts the passing of hydrophobic drugs [15], so Naringenin's therapeutic potential is constrained by its lipophilic nature, which results in poor bioavailability [16].

The shortcomings of current ophthalmic drug application technologies have been beneficially resolved by the invention of ophthalmic drug delivery systems, especially nano-scale systems. These routes of administration can improve the ability of flavonoids to access the internal structures of the eye and their ocular bioavailability [17].

Liposomes may encapsulate both hydrophilic and hydrophilic medicinal molecules and are biodegradable, biocompatible, and non-toxic carriers. In addition, liposomes can be used to deliver drugs to the eyes [18]. They are able to prolong the time a drug is in contact with ocular tissue, enhance the absorption of drug, and improve ocular bioavailability as well as patient satisfaction due to a decrease in the dosing rate. In liposome systems, drugs can be shielded from the action of enzymes in the corneal epithelium or tear film, which lowers the formulations' clearance rate [19]. This study aimed at the development of an effective liposome for Naringenin topical ocular administration. Hence, we studied for the first time the influence of prepared NLL as topical ocular drug delivery on the markers of oxidative stress in type 1 diabetic rat's lenses to prevent or delay the diabetic cataractogenesis.

2. Materials and Methods

2.1. Chemicals

The following substances: Naringenin, lecithin and Cholesterol (Sigma Chemical Company, USA), Streptozotocin (Tuko Company), Phosphate buffer saline (Nasr Pharma Company), EDTA (Advent Company), TCA (dop-ORGANIK sentex Company), [Ellman's reagent, sodium carbonate and sodium hydroxide (adwic Egypt Company)**],** Folin ciocaltu reagent (Fisher scientific UK Company). IL6 and Caspase 3 Kits (SunLong Biotech Co, China) and VEGF (BT-Lab Company, China) were used in the current study. The molecular structure of Soy-lecithin liposomes and Naringenin are shown in **Figure 1**.

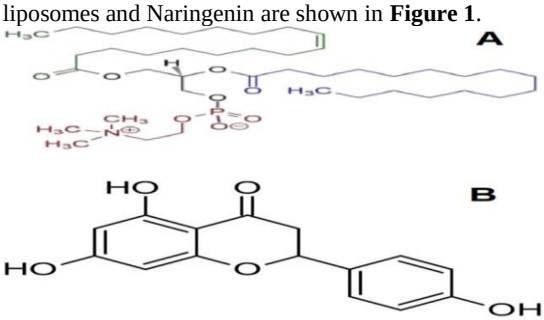


Fig.1. Chemical Structure of Soy-lecithin liposomes (A), and Naringenin (B)

2.2. Experimental animals.

This work was conducted on 60 male Wistar rats, of weight about 150-180 grams and received from the laboratory animal research house, Research Institute of Ophthalmology (RIO). The experimental animals were kept in stainless steel cages with food and water supply. Animals were preserved in stable environments of aeration, temperature, moisture (60-70%) and light/dark condition (12/12hrs). Animal care and protocols were according to the guidelines and approval of the Ethical Committee of Research Institute of Ophthalmology (RIO). The rats eyes were examined using a slit lamp (Carl Zeiss) before diabetes induction and those with any defect of lens were excluded.

2.3. Naringenin liposome preparation as eye drops

Soybean lecithin: Naringenin molar ratio 7:2 was utilized to formulate neutral multilamellar vesicles (MLVs) by the technique of [20]. In a 100 ml flask, 2.41 mg of Naringenin powder and 20 mg of lecithin were transferred quickly. At a molar ratio of 1:7, cholesterol was put to the lipid composition. The flask was then shaken while 20 ml of ethanol was added, ensuring that all of the lipids had dissolved. A thin dry layer of lipid was created when the solvent was evaporated under vacuum using a rotary evaporator. The flask was then placed under vacuum for a further hour to guarantee that all traces of ethanol had evaporated. Following that, 10 ml of buffer (10 mM Trizma adjusted to pH 7) was put to the flask. A nitrogen stream was then flashed across the flask, and it stoppered immediately. For 0.5 hour, the flask was shaken mechanically at a temperature greater than 50 ºC. Following the same conventional procedures as previously described, control empty liposomes were created using only aliquots of 20 mg of soybean lecithin. According to calculations, there are 0.241 mg of Naringenin per milliliter of Trizma buffer.

2.3.1. Liposome morphology by TEM

A negative stain transmission electron microscope HR-TEM (Tecnai, G20, FEI, Netherlands) operating at 200 kV was used to analyze the size and shape of empty and liposomes containing Naringenin encapsulated. A negative staining agent, phosphotungstic acid stain in an aqueous solution (1% w/v) was utilized. A 20 µl aliquot of the liposome samples was then applied to a TEM grid (carbon-coated copper grid) after being diluted (1:10) in Tris buffer pH 7.4 at 37°C. The solution was then left for 1 minute, and the filter paper was used to extract the excess from the grid. TEM images were taken and analyzed

2.3.2 DLS and Zeta potential

Using the "Nanotrac Wave II, Microtrac, USA" (particle sizing method) for dynamic light scattering (DLS) in 7.4 PH of tris buffer at 25 °C, the zeta potential, mean particle size, and size distribution of liposomes loaded with Naringenin and blank liposomes were calculated. All of the triplicate data were expressed as mean ± St. Dev.

2.3.3. DSC

Experiments are conducted to examine the temperature performance of empty and Naringenin-loaded liposomes lyophilized samples using DSC (model DSC-50, Shimadzu, California, USA) standardized with indium. 5-mg sealed samples are analyzed in standard aluminium pans. The thermogram for each sample scans at a rate of 3°C/min throughout a temperature range of 25 to 200°C.

2.4. Experimental Design and diabetes mellitus induction

Forty rats fasted until the next morning and received a solitary injection (intraperitoneally) of streptozotocin (STZ) at a dose of 55 mg/kg rat weight, liquefied in 0.1 mol/L citrate buffered solution (pH 4.5) to induce diabetes as stated by [21]. After 3 days of STZ injection, fasting blood sugar (FBS) was monitored and rats with FBS greater than 200 mg/dl have been considered as

diabetic. Experimental rats were distributed into three groups:

Group I: control group (20 rats); Rats received a solitary intraperitoneal injection of streptozotocin-vehicle-containing citrate buffer in accordance with their body weight

Group II: diabetic group (20 rats); Diabetic rats left untreated.

Group III: Naringenin treated group (20 rats); each animal received one drop of naringenin liposome formula instilled into the eye twice a day for twelve weeks.

2.5. Ophthalmological examination

Throughout the study period, ophthalmological examinations of all animals' eyes were performed once a week. Before performing a fundus examination, single drop of tropicamide 1% mydriatic was installed into the eyes of rats at least 30 minutes beforehand. To confirm pupil dilation, another drop was administered if necessary. The animals were given 20 mg/kg of ketamine to anaesthetize them into the rat's thigh muscle ten minutes before the fundus examination; the rat's eyes were then given one drop of a local anesthetic with proparacaine hydrochloride 0.5%. During lengthy tests, more ketamine (5 mg/kg) was given as needed. Dark room along with an indirect ophthalmoscope was used to examine the fundus (Heine and 20 D Volk lens).

2.6. Morphological assessment of cataract

Slit lamps and bio-microscopes were used for lens inspection and photography (Horvitz, HS500, Shanghai, China). The grading of cataract changes was done independently by three observers that were unaware of the grouping of animals. Considering the cataract ophthalmic assistants grade lens opacity on a scale from 0 to V based on the ophthalmoscopic examination as [22]:

a -Stage 0: - represent clear lens

b- Stage I-II: - represents mild opacification

c -Stage III-IV: - represents moderate opacification

d- Stage V: - represents complete lens opacification

2.7. Blood and lens tissue collection.

At the end of the research, blood was drawn to estimate the glucose level. Animals were scarified by over dose of diethyl ether and the eye balls were removed from rats and perfused with saline to remove blood cells and clots. Lenses were separated from the eye balls using a sharp blade. The weight of the lenses was measured using a small clean glass tube and kept at -70^{o}C for further analysis.

The collected lenses were used to create two distinct homogenates, one from each group. The first homogenate was 7% made from the grinding of one lens in 10% TCA, centrifuged at 3500 rpm for 30 min., and the obtained supernatants were utilized to investigate the reduced glutathione in the lenses. The second homogenate was 20% made from the grinding of three pooled lenses in phosphate buffer saline pH 7.4.

By utilizing a cooling centrifuge (Centric200R, Domel-Tehtnica, Slovenia), the homogenized tissue was centrifuged for 20 minutes at 12,000 rpm and 4^{o}C. In order to measure total soluble lens protein, carbonyl protein, caspase-3, VEGE, and IL6 in the lens, the supernatant was taken out and preserved in aliquots at -80^{o} C.

2.8. Assay of glucose level

Using a kit from Bio Diagnostic and the procedure described in [23], plasma glucose was measured enzymatically.

2.9. Lens reduced glutathione assay

According to a technique developed by [24] and modified by [25], reduced glutathione levels were assessed in lens tissue. 0.2 ml of supernatant was inserted to 0.59 ml of Tris-HCl buffer and 0.01 ml Ellman's reagent, then 0.04 M EDTA was added to make 1 ml. Spectro UV-visible double beam was used to measure the absorbance of sample at 412 nm. The concentration of GSH was obtained by the relation:

$$\text{GSH in Lens } (\mu\text{mol/g.lens}) = \frac{\text{conc from st.curve } (\mu\text{mol/ml})}{0.2 \times 307 \times \text{ g tissue used}}$$

Where

0.2 refers to volume used from the sample by ml

307 refers to the molecular weight of reduced glutathione

2.10. Assay of total soluble protein (TSP) in lens:

The method of [26] was utilized to investigate TSP content of the lens. 5 µl of standard samples were taken from the lens homogenate, completed to 1 ml with dist. water. 5ml of alkaline copper reagent was inserted. Then allowed to stand for 10 mins, and then 0.5 ml of diluted foline reagent was added. The solution was allowed to stand for 30 minutes. A blank solution composed of 1 ml distilled water and 5 ml mixed alkaline copper reagent was left for 10 minutes and 0.5 ml diluted foline reagent. OD was measured using a spectrophotometer (UVD-3200) at 710 nm. The standard curve was utilized to calculate the protein concentration in the sample, which was then represented as mg/g wet wt.

2.11. Assay of Carbonyl Protein (CP) in lens

CP was determined according to [27]. 200 µl of lens protein samples were mixed with 800 µl of 10 mM DNPH in 2.5 M HCl, then incubated for 1 h at dark room temperature and vortexed. 1ml of 20% (w/v) Trichloro-acetic acid (TCA) was inserted prior incubation on ice for 5 min, next centrifugating at 8000 r.p.m for 10 min at 4^{o}C, the supernatants were gathered and blended with 1 ml of 10% (w/v) TCA prior centrifugation at 8000 r.p.m for 10 min at 4^{o}C. The protein granules were washed 3 times with 1 ml of 1:1 (v/v) ethanol: ethyl acetate and centrifuged at 8000 r.p.m for 10 min at 4^{o}C. Once last washing, the protein pellet was suspended again in 500 µl of 6 M guanidine hydrochloride and centrifuged at 8000 r.p.m for 5 min at 4^{o}C.The supernatant were gathered and measured at 370 nm using control as a blank. Using the equation:

Carbonyl Protein µmol /ml = (A370/0:022 µM - 1) (500 µl /200µl)

22mM/cm is the extinction coefficient for aliphatic hydrazone. Carbonyl protein in lens was expressed as µmol /mg protein.

2.12. Assays of IL6, Caspase-3 and VEGF in lens tissue

According to the manufacturer's instructions, IL6 and Caspase 3 levels in the lenses were measured using SunLong Biotech kits (Biotech Co., LTD, Zhejiang, China), and VEGF was measured using a BT-Lab kit (BT-laboratory, Zhejiang, China).

2.13. Statistics

Results weResults were presented as the mean ± S.D. Data were examined using a one-way analysis of variance (ANOVA). Post hoc analysis using the least

significant difference was used to compare the differences between the groups (LSD). Statistics were considered significant when the P-value was less than 0.05.

3. Results

3.1.Naringenin liposome eye drops preparation results

3.1.1. Liposome morphology by TEM

As shown in **Figure 1**, all prepared liposomes morphology was spherical in shape, well scattered and less aggregated for empty (**Fig. 2A**) and encapsulated vesicles. Findings from TEM demonstrated a physical relationship between naringenin and the disruption of liposomes' surface membrane packing property (**Fig. 2B**). In comparison to the controls, the liposomes were larger because the presence of naringenin increased the spacing between adjacent bilayers.

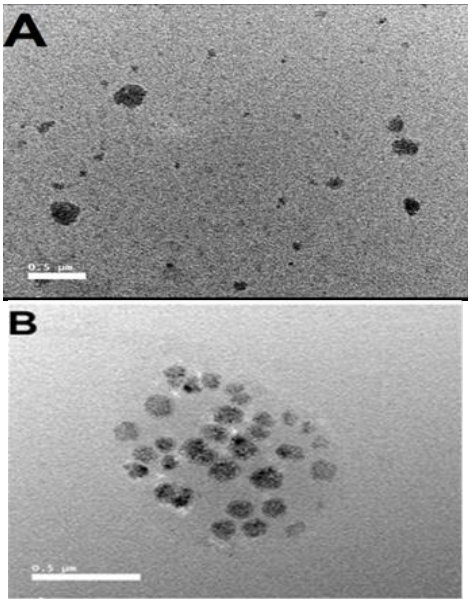


Fig.2: TEM images for empty liposomes (A), and Naringenin - loaded liposomes (B)

3.1.2. Liposomes size distribution by DLS

Colloidal suspension particle homogeneity is effectively accounted for by the poly dispersity index (PDI). Values greater than 0.7 indicate that the sample has a large size variation make DLS method was unstable. The size distribution of a pure soy lecithin lipid sample is depicted in **Figure (3A)** as being concentrated around a mean size diameter of 152.1 ± 99.70 nm with 0.333 PDI. Following the encapsulation of naringenin into pure soy lecithin lipid, **Figure (3B)** shows a rise in the mean size diameter of pure soy lecithin to 153.5±118.4 nm with 0.279 PDI.

3.1.3. The zeta potential magnitude

As shown in **Figure 4**, empty liposomes displayed negative zeta potential of magnitude (-41.3±8.31 mV). Because Naringenin was integrated into the liposomal membranes, Naringenin-loaded liposomes displayed higher negative zeta potential (-48.5±6.05 mV) than blank liposomes.

3.1.4. DSC results

Soy lecithin vesicles were used as a model membrane as this phospholipid may mimic many aspects of biological membranes. When submitted for DSC analysis, pure soy lecithin vesicles showed a large major endothermic peak (T_m) at 73.92 °C. **Figure 5** illustrates how the temperature of soy lecithin liposomes after naringenin was added increased to 93.4 °C from the major endothermic peak (T_m) of empty soy lecithin, which is present at 73.92 °C.

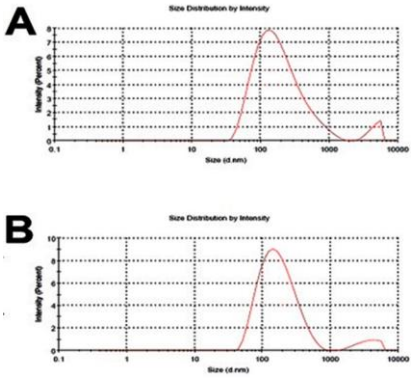


Fig.3: Liposomes size distribution measured by dynamic light scattering (DLS). (A) empty Soy lecithin liposomal sample, and (B) Naringenin - encapsulated liposomes.

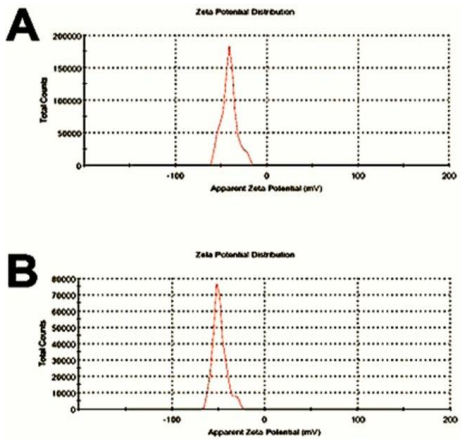


Fig.4: Zeta potential for (A) empty Soy lecithin liposomal sample, and (B) Naringenin -encapsulated liposomes.

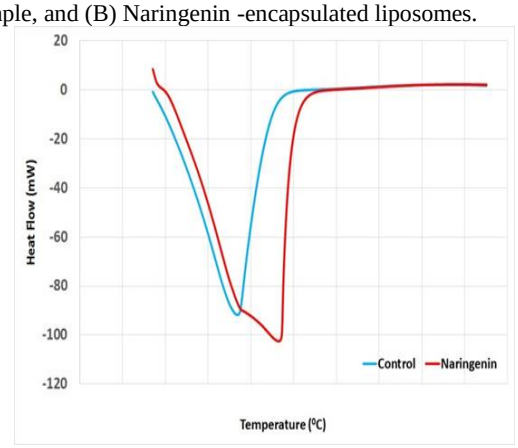


Fig.5: DSC diagrams of liposomes made of empty soy lecithin; liposomes doped with Naringenin.

3.2. Fasting blood sugar level

Comparing with control rats, the FBS values in the diabetic group was significantly higher, Naringenin treated group revealed further increase in blood glucose concentration compared with diabetic group, the percent of changes of F.B.S concentration from the N control group were 75.5% and 102.2% in the diabetic, and Naringenin treated groups respectively. Also, the percent of change of

F.B.S concentration from diabetic group was 15.18% in Naringenin treated group as shown in **Table (1)**.

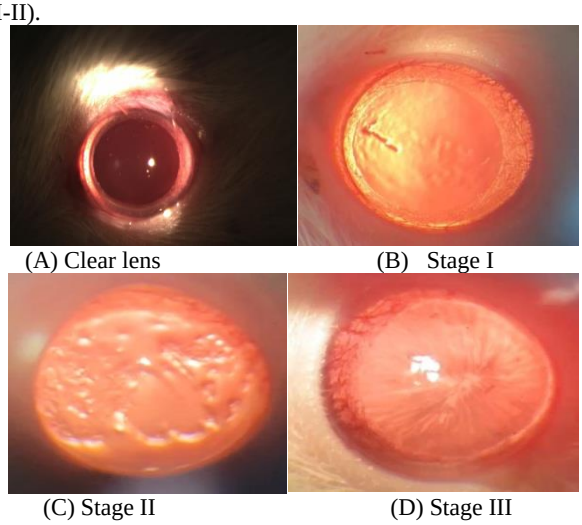
Table 1: The mean values of fasting blood glucose in different studied groups

Group \ Parameter		F.B.S (mg/dl)
Group 1 N. control	**Range Mean ± SD**	**72.4 – 104.3 78.9 ± 13.9**
Group 2 Diabetic	**Range Mean ± SD % Change**	**159- 245.2 201.9 ± 24.9 [b] ↑155.9%**
Group 3 Naringenin Treated	**Range Mean ±SD % Change % Change***	**151 – 214.8 184.7 ± 19.6 [b,d] ↑134% ↓- 8.5%***

% Change vs control group
%change* vs diabetic group
[a]: $P < 0.05$, [b]: $P < 0.001$ vs. N. control group
[c]: $P < 0.05$, [d]: $P < 0.001$ vs. Diabetic group
$p > 0.05$ is non- significant, $p \leq 0.05$ is significant, $p \leq 0.001$ is highly significant

3.3. Progression of Cataract

At the end of the research, **Figure (6)** displayed the representative photographs of lenses in various phases of diabetic cataract. **Table (2)** showed slit lamp examination results, all the lenses in control group were clear and normal (stage 0), about 10 % of the diabetic rats represented clear lenses (stage 0), and about 15% represented mild degree of cataract (stage I), 45% in (stage II) represented mild opacification. In addition, about 30% of the diabetic rats represented moderate opacification (stageIII). However, in treated rats most of the lenses were in stage 0 (60%) and only 40% of the lenses were represented mild opacification (stage I-II).



(A) Clear lens (B) Stage I
(C) Stage II (D) Stage III

Fig.6. Representative Photographs of lenses showed different stages of diabetic cataract at the end of the experiment.

3.4. Biochemical parameters in lens tissue

3.4.1. Total soluble lens protein (TSP in lens)

As shown in **Figure 7**, there was a high significant decline in the total soluble lens protein in both diabetic and Naringenin treated groups compared with normal control, but Naringenin treated rats revealed a highly significant rise in this parameter compared to diabetic group.

Table 2: Percent of different stages in diabetic cataract and clear lens in all experimental groups

Group	Stage of Cataract			
	Stage (0)	**Stage (I)**	**Stage (II)**	**Stage (III)**
N. Control group (n=20)	20 (100%)	0	0	0
Diabetic group (n=20)	2 (10%)	3 (15%)	9 (45%)	6 (30%)
Naringenin treated group (n=20)	12 (60%)	7 (35%)	1 (5%)	0

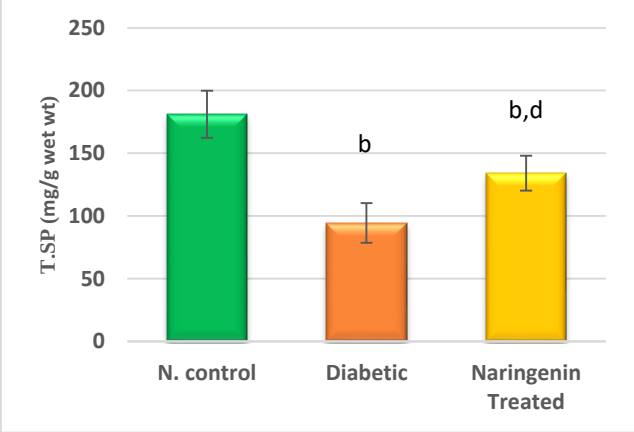


Fig.7: Total soluble lens protein in experimental groups. Results are presented as arithmetical means ± SD. The letters (a–d) above the bars indicate statistical significance (one-way ANOVA followed by Fisher's LSD test). a: P < 0.05, b: P< 0.001 vs. N. control group. c: P< 0.05, d: P< 0.001 vs. Diabetic group.

3.4.2. GSH and Carbonyl protein (CP) in lens:

Figure (8) showed that, both diabetic and Naringenin treated groups resulted in a significant decline in lens GSH level comparing with control, but when comparing Naringenin treated rats with diabetic ones, the level of GSH exhibited a high significant increase. Diabetic group showed a highly significant increase, and Naringenin treated group exhibited no significant changes in lens carbonyl protein levels compared to control group. Whereas Naringenin treated group exhibited a high significant decrease in CP compared to diabetic group (**Figure 9**).

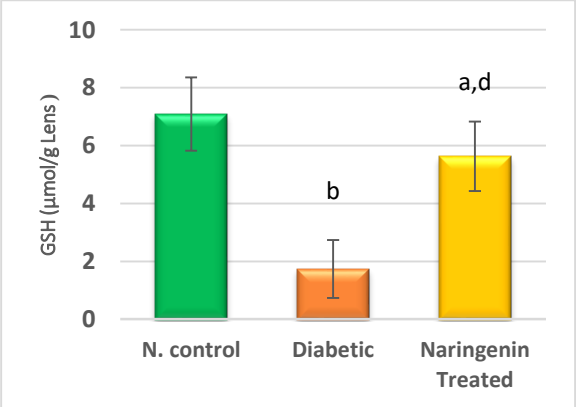


Fig.8: GSH of lens tissue in experimental groups. Results are presented as arithmetical means ± SD. The letters (a–d) above the bars indicate statistical significance (one-way ANOVA followed by Fisher's LSD test). a: P < 0.05, b: P<

0.001 vs. N. control group c: P< 0.05, d: P< 0.001 vs. Diabetic group

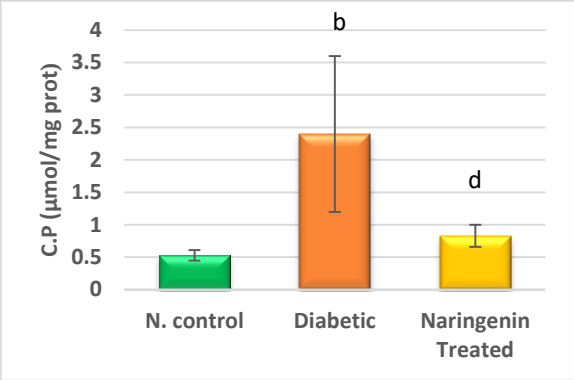


Fig.9: Carbonyl lens protein in experimental groups. Results are presented as arithmetical means ± SD. The letters (a–d) above the bars indicate statistical significance (one-way ANOVA followed by Fisher's LSD test). a: P < 0.05, b: P< 0.001 vs. N. control group c: P< 0.05, d: P< 0.001 vs. Diabetic group

3.4.3. Level of Caspase-3 as apoptotic marker in lens tissue:

There was a high significant elevation in lens caspase-3 level as comparing the diabetic group with control one, and a non-significant change was noticed when the Naringenin treated group compared to control. Naringenin treated animals showed improvement in lens caspase-3 level compared to diabetic rats (**Figure 10**).

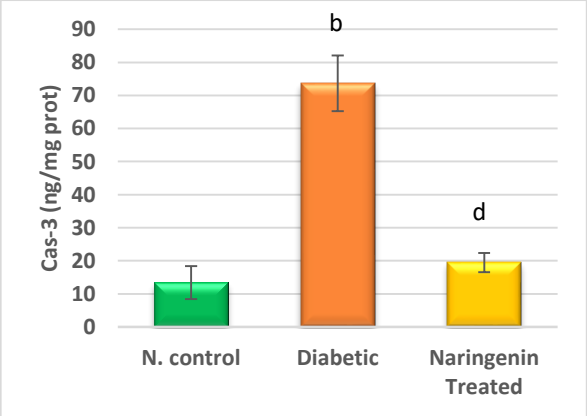


Fig.10.Caspase-3 levels of lens tissue in experimental groups. Results are presented as arithmetical means ± SD. The letters (a–d) above the bars indicate statistical significance (one-way ANOVA followed by Fisher's LSD test). a: P < 0.05, b: P< 0.001 vs. N. control group. c: P< 0.05, d: P< 0.001 vs. Diabetic group

3.4.4. Inflammatory (IL6) and Angiogenic (VEGF) biomarkers in lens tissue:

Figure (11) demonstrated that diabetic and naringenin treated groups induced a marked elevation in lens levels of IL6 as compared to control group. Naringenin treated group exhibited a highly significant reduction in Il-6 level compared to diabetic group. High significant rise was detected in VEGF level in both diabetic and Naringenin treated groups as compared to control while a highly significant reduction in the level of VEGF was determined in Naringenin treated group when compared to diabetic rats as shown in **Figure (12)**.

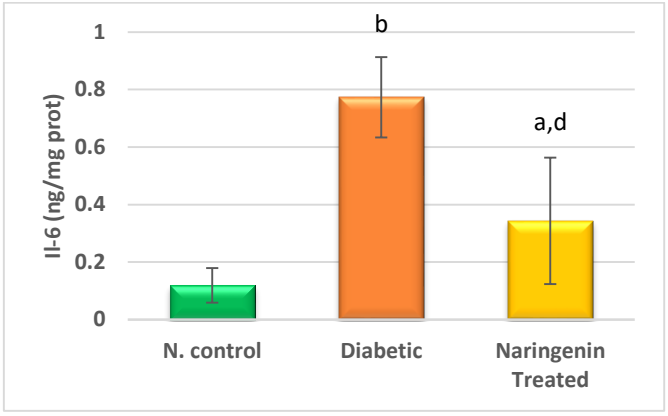


Fig.11: IL-6 levels of lens tissue in experimental groups. Results are presented as arithmetical means ± SD. The letters (a–d) above the bars indicate statistical significance (one-way ANOVA followed by Fisher's LSD test). a: P < 0.05, b: P< 0.001 vs. N. control group. c: P< 0.05, d: P< 0.001 vs. Diabetic group

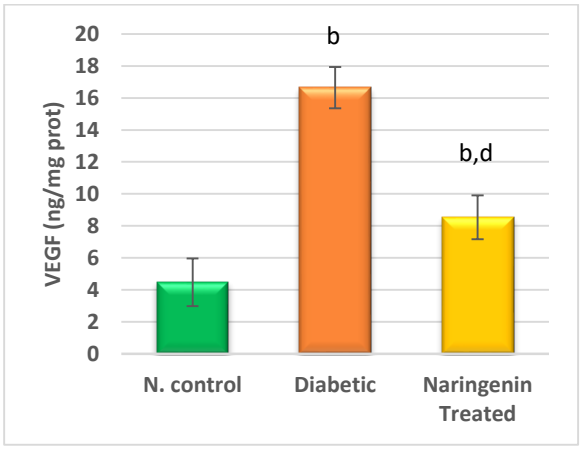


Fig.12: VEGF levels of lens tissue in experimental groups. Results are presented as arithmetical means ± SD. The letters (a–d) above the bars indicate statistical significance (one-way ANOVA followed by Fisher's LSD test). a: P < 0.05, b: P< 0.001 vs. N. control group c: P< 0.05, d: P< 0.001 vs. Diabetic group

4. Discussion

Type 1 diabetes mellitus (T1DM) leads to pancreatic beta cells damage and it is considered as an autoimmune condition. Insulin is a crucial anabolic hormone that influences growth as well as the metabolism of glucose, lipids, proteins, and minerals [28]. Uncontrolled hyperglycemia can lead to many ocular complications. Additionally, it causes a rise in reactive oxygen species (ROS), which in turn causes intercellular structures like proteins and lipids to oxidize and become damaged. Thus lead to cataract formation and lens opacity [29]. As a result, the usage of antioxidative substance in the treatment of diabetic ocular complications seems reasonable. Numerous substances derived from plants have so far been touted as effective treatments for diabetic rats' lens changes caused by oxidative stress [30]. One class of these phytochemicals

is flavonoids, which have been extensively studied as multifunctional mediators in the inhibition of diabetic cataract [31]. Flavonoids are strong antioxidants.

In animal models, naringenin has been shown to have advantageous effects on ocular structures. Naringenin protects ocular neurons from deterioration and slows down the aging of the retinal pigment epithelium when applied topically [32]. Naringenin's hydrophobic nature causes a very low in vivo bioavailability, which restricts its usefulness. It has a brief half-life and quickly transforms into its crystalline form, which has a poor capacity for absorption [33]. Numerous initiatives have been made to increase naringenin's bioavailability as a result of its usage restrictions. Use of delivery systems at the nanoscale range is one of the most promising candidates. Every delivery method has unique advantages and disadvantages for various applications. Because of their structural characteristics, liposomes have several distinct advantages over other delivery systems, including strong biocompatibility, the ability to deliver both hydrophilic and hydrophobic nutraceuticals, desirable encapsulation efficiency, and the capacity to transport across cell membranes [34].

Transmission electron microscopy (TEM), dynamic light scattering methods (DLS), and differential scanning calorimetry are used to assess the physicochemical characterizations of the liposomal system (DSC). Our current strategy was justified by the need to extend Naringenin's ocular contact time through the use of liposomes. We show that the morphology of every liposome that was created was almost spherical, and that size is a crucial physical element. The bioavailability of the medicine that is entrapped is improved by nanomaterials and nanodrug delivery systems because of their size compatibility with cell dimensions. It is common knowledge that size distribution is a significant issue in the use of liposomes in pharmaceuticals.

It is regarded as a crucial factor in describing the liposome suspensions' quality. They also serve as crucial indicators of the biological fate of liposomes and the substances they contain in vivo, as well as their physical characteristics. For parenteral administration, for instance, the ideal size range is between 70 and 400 nm. Liposomes favor the accumulation of drugs into specific target organs, such as the liver, spleen, and bone marrow, within this size range. They also exhibit predictable drug-release rates due to their good blood stability.

Liposomes made with naringenin were larger in size than those made with controls because it increased the distance between adjacent bilayers (**Figure 2B**). Stronger drug interactions with the lipid bilayer of liposomes through hydrogen bonding may be the cause of the rise in particle size. These findings agree with the data shown by the DSC results, which supports the hypothesis that Naringenin might be enclosed in the hydrophobic portion of the bilayer.

Since Naringenin is primarily thought of as a lipophilic drug and might be trapped in the hydrophobic center of the bilayer, the encapsulation of Naringenin into pure soy lecithin lipid lead to rise in the mean size diameter of pure soy lecithin. This was most likely caused by the electrostatic repulsion force between the negative charge of soy lecithin PO_2^- group and the negative charge of Naringenin OH^- group. When Naringenin was included to liposomes, the distance between neighboring bilayers was increased, resulting in the development of liposomes that were larger than the control ones. These results propose that Naringenin may be physically incorporated into liposomes, and that the liposome's molecule has a strong tendency to interact with the lipid bilayer and disturb it.

An indication of the colloidal system's potential stability can be found in the zeta potential's magnitude. The more stable the colloidal dispersion is, the more strongly the particles would repel one another as the zeta potential rises. The particles won't have a tendency to combine if all of the suspended particles have a strong negative or positive zeta potential [35]. The inclusion of Naringenin appears to have made the zeta potential negative by increasing the density of negative charge.

Among the most popular methods for examining drug-biomembrane interactions are calorimetric methods. For these experiments, DSC is a technique used. When a medication molecule is present in a liposome sample, thermotropic changes eventually take place that can be measured by DSC. The fundamental tenet of DSC is that, as temperature rises, phospholipid vesicles go through a reversible phase transition from a "gel" state, in which the acyl chains are neatly packed within the bilayers, to a "liquid crystal" state, which is characterized by an increase in spatial disorder in the bilayers. A transition like this is coupled with heat absorption, making it an endothermic process. The pre-transition temperature (T_p), which mostly relates to the polar region of phospholipids, is the temperature at which a transition from the gel phase to the rippling phase occurs. Then, at the major transition temperature, the bilayer melts from the rippled to the liquid phase (Tm). The melting point represents the endotherm peak temperature for the lipid gel-to-fluid phase transition observed during the heating scan (T_m). Changes in the lipid structure have a significant impact on all of the aforementioned phases.

Naringenin had a significant impact on the soy lecithin bilayer of acyl chains, producing a conformational regularity within the phospholipids and increasing the cooperative transition of lipid acyl chains, as shown by a shift to a higher temperature following the addition of Naringenin to soy lecithin liposomes comparing with the main endothermic peak (T_m) of empty soy lecithin [36,37]. The primary endothermic peak (T_m) of empty soy lecithin increased in temperature, indicating that the incorporation of Naringenin makes it easier for acyl chains to form in an ordered and tight state.

As revealed by biophysical/biochemical examinations liposomal Naringenin have advantages over the solution dosage form:

(a) Lower dose concentration of Naringenin.

(b) Better prognosis decreasing the period of treatment.

(c) Lower dose frequency increasing the patient compliance.

(d) Liposomal Naringenin are interacting strongly with lipid membrane.

(e) The deeper comprehension brought about by this study may open the door to optimizing the use of naringenin-loaded liposomes in liposomal drug delivery systems and utilizing them to specifically engineer the properties of cell membranes and other biologically significant lipid layers. Future research will assess the pharmacokinetics, biodistribution, and effectiveness of naringenin-loaded liposomes in vivo in order to improve their therapeutic applications.

(f) According to biophysical/biochemical tests, we suggest that naringenin-loaded liposomes will be effective as a nanomedicine with diagnostic and therapeutic abilities because of the dual drive to lessen the toxicity and side effects of current treatments and increase efficacy by specifically targeting the eye.

On the basis of this study, it seems that liposomes are efficient carriers for Naringenin and have a valuable capacity as drug delivery techniques in the ocular therapy.

In the current research, type 1 diabetes was generated in wistar rats by injecting them with streptozotocin (a single dose of STZ at 55 mg/kg body weight) [38] to induce type 1diabetes in both diabetic and treated (by local delivery with Narengenin liposome eye drops) groups. According to Yin *et al* [39] A single STZ injection can cause rodents to develop type 1 diabetes.

In this study, it is interesting to note that fasting blood sugar levels were the highest in diabetic and treated (by local delivery with Narengenin liposome eye drops) rats compared to control group during experimental period; this was because the local administration of Naringenin as eye drops (in Naringenin treated group) not give high effect on the level of blood glucose, this result agree with that of [40].

The development of diabetic cataracts is a complicated process involving oxidative stress, activation of the polyol pathway, and protein glycation [4]. Since oxidative stress is one of the primary factors that contribute to the development of diabetic cataracts and cataract surgery may be related with an increased risk of postoperative complications in diabetic patients, it is necessary to ascertain whether any antioxidant compounds have the potential to slow down or stop the development of cataracts. Antioxidant activities of many polyphenolic compounds originating from plants have been established [30].

Our ophthalmic examination results show that Naringenin was able to stop the lens from becoming opaque. Because most lenses in rats treated locally with liposomal preparations of naringenin eye drops (60%) were in stage 0 and only 40% had mild opacification (stage I-II), This finding is consistent with those of Patil et al. [41], who found that Naringenin had an impact on glycation-induced lens opacity and that flavonoids were effective at inhibiting the glycation reaction and reducing diabetic-induced cataractogenesis.

The natural lens of the eye becomes opaque as a result of cataract development, which reduces visual acuity. Transparency is a result of lens proteins' normal soluble phase, which accounts for their existence. Certain conditions, including aging, diabetes, steroid use, and trauma, cause proteins to exit the soluble phase and form high molecular weight aggregates, which are responsible for the lens's opacity and light scattering [42]. Since it causes DNA fragmentation, lipid peroxidation, and protein modifications, all of which are thought to contribute to cataract formation, oxidative stress seems to play a significant role in this cascade of molecular reactions [43].

Low light scattering must be preserved to guarantee lens transparency. As a result, lens cells only contain specialized proteins known as crystalline and lack organelles [44]. Because of the aggregation of crystallins under diabetic conditions, there is a decline in soluble protein level and an increase in light scattering [45].

Changes to lens proteins and insolubilization are thought to be the main factors contributing to lens opacification during cataract formation. Consequently, we investigated the total soluble protein content of lenses from all groups. Both STZ diabetic and Naringenin-treated rats in our study showed a decrease in total soluble lens protein when compared to control, but Naringenin-treated rats exhibited a highly significant rise in this parameter as compared to the STZ diabetic group. This finding is consistent with that of [46], who suggested that a partial leakage of proteins into the aqueous humor may be the cause of the decrease in lens soluble protein. Naringenin administration to the rats prevented the injury of all soluble protein from the lens, and this resulted in a delay in the development of cataract in this group.

We evaluate the level of soluble protein and carbonyl protein to assess oxidative damage in proteins. The development of oxidative stress and protein dysfunction are thought to be connected to the accumulation of carbonyl protein [47]. In this study, the STZ diabetic group had significantly higher levels of lens carbonyl protein than the control group. As compared to control rats, the naringenin-treated group showed no discernible differences in this parameter; while a highly significant decrease was observed when comparing the Naringenin-treated group with the STZ diabetic one. Amino acids in oxidized proteins may directly interact to form the lens carbonyl protein. It is possible that the decrease in the CP level was related to the decrease in ROS level in the lens environment. This finding is in agreement with Wojnar et al. [48] who found that naringenin decreased the oxidants level in diabetic rat's lenses.

Diabetic cataracts can occur as a result of reduced glutathione (GSH) deficiency in the lens impairing antioxidative mechanisms [49]. It is well recognized that maintaining GSH is essential for lenticular transparency because it functions as an essential intra- and extracellular defensive antioxidant opposed to oxidative stress. It has a side chain sulfhydryl (-SH) residue that gives it the ability to defend cells opposed to oxidants [50].

In our research, it was exhibited that the level of GSH was significantly smaller in the lenses of diabetic rats than that in normal rat, these results agree with that of Wojnar *et al* [48]. While Naringenin treated animals showed a significant rise in lens GSH parameter compared to diabetic rats and this is in accordance with Hilliard *et al* [51] who revealed that Naringenin as a flavonoid has antioxidant effect increasing GSH in lens.

Apoptosis represent a kind of cell death which aids to remove dying cells in differentiating cell populations. Therefore, it plays a vital role in the cell development and tissue homeostasis [52]. It was revealed that apoptosis of lens epithelial cells has critical function in the progression of different kinds of cataracts [53]. Caspase-3 is stimulated in the early stages of apoptosis after cataract formation [54]. In the lens epithelium of the cataract, inhibition of apoptosis can postpone cataractogenisis.

In our study there was a high significant increase in the level of caspase-3 in lens in diabetic group compared to control one and a non-significant change in the level of caspase-3 when Naringenin treated group compared to control group. Naringenin treated animals showed improvement in lens caspase-3 compared to STZ diabetic rats. In our opinion, the inhibitory effects of naringenin on

diabetic –induced apoptosis may be as a result of their antioxidant chrarcteristics and their aptitude to scavenge free radicals these results agree with that of Osama *et al* [55] who stated that naringenin may exert its hepatopreventive effects in N-acetyl-p-aminophenol-administered rats through improvement of the antioxidant defense system and repression of inflammation and apoptosis.

A crucial cytokine that is substantially up-regulated during infection and inflammation and linked to a number of systemic autoimmune disorders is IL-6. Numerous ocular diseases, including glaucoma, dry eye disease, corneal infections, and ocular inflammatory diseases, have been linked to raised levels of IL-6 [56]. Through the activation of vascular endothelial growth factor (VEGF), lipid peroxidation, protein and DNA damage, and apoptosis, which cause cataract and diabetic retinopathy glaucoma, oxidative stress plays a vital role in age-related eye diseases pathogenesis [57]. VEGF is induced when there is hypoxia, and IL-6 is crucial for this process. IL-6 and VEGF levels in the vitreous were linked with disease severity [58].

In the present work, STZ diabetic and treated groups induced a marked elevation in lens levels of Interleukin-6 as compared to control group. Naringenin treated group exhibited a highly significant decrease in lens Il-6 level compared to diabetic group. This result matches with that of Oguido *et al* [59]. Also, High significant increase was observed in lens VEGF level in both diabetic and treated groups as compared to control, while a highly significant reduction in the level of lens VEGF was determined in treated rats when compared to diabetic rats.

5. Conclusion

In our study we prepared biologically active nanoformulation of Naringenin liposomal ocular drug. The felicitous formulation of Naringenin liposome was confirmed by TEM imaging. The particle size, zeta potential and DSC results disclosed a good value for flavonoid Naringenin as a lipophilic compound. Naringenin liposome exhibited antioxidant efficacy in many oxidative stress-related parameters in the lens tissue of diabetic rats. Also, the ameliorative effect of this formula on the levels of Caspase-3, IL6 and VEGF as apoptotic, inflammatory and angiogenic biomarkers respectively, in diabetic cataract rats was assessed.

The strong point of the present work can be gotten as its novelty in the preparation of Naringenin liposomal nanoparticles eye drops and its influence on diabetic cataractogenesis. Also, confirming the antioxidant property of the prepared liposomal formulation of Naringenin on lens tissue affected by oxidative stress due to diabetes. However, limitations were observed in the current work as not investigating the cytotoxicity of NLL prepared.

6. Conflict of interest: No potential conflict of interest relevant to this article was reported.

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