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RESEARCH ARTICLE

Formulation, Characterization and Toxicity Assessment of Ginkgo Biloba Extract Solid Lipid Nanoparticle in female mice

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ABSTRACT

Objective(s): We aimed in present study to provide solid-lipid nanoparticle (SLN) of GBE and evaluate its oral safety in the light of recent discoveries on strong inhibitory effects of Ginkgo Biloba Leaf Extract (GBE) against Covid 19 and Influenza virus.

Methods: Morphology and particle sizes of nanoparticles were analyzed by Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) and Dynamic Light Scattering (DLS) methods. In the next step, the release profile of 85% loaded SLNs was determined by dialysis membrane method. Acute and repeated dose oral toxicity tests were performed on selected freeze-dried formulation with appropriate stability in female mice according to OECD 425 and OECD 407 guidelines.

Results: The spherical GBE-SLN released GBE during the first 72 hrs. In acute oral test, doses up to 2000 mg/kg didn't cause mortality or any sign of toxicity .Repeated dose oral toxicity study on three dose levels (0.5, 5, 50mg/kg/ day), didn't show any abnormal change due to clinical, biochemical and necropsy evaluations but hematological assessment showed coagulative abnormalities in accordance with some abnormal changes in the liver, kidney, heart, lungs and ovaries of high dose (50mg/kg) group of animals.

Conclusions: Repeated dose oral administration of GBE-SLN in doses up to 5 mg/kg/day was considered as safe dose level. Later studies are mandatory for evaluating the preventive and therapeutic effects of present GBE-SLN against Covid 19 in appropriate in vitro and in vivo models.

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INTRODUCTION

The originally Chinese tree of Ginkgo biloba (ginkgo), is one of the oldest and still living species of trees, which is cultivated around the world as an ornamental tree but many medical applications have been determined for Ginkgo biloba leaf extract (GbE) which caused its continued use for centuries around the world (1) . Nowadays the major constituents of Ginkgo biloba leaf extract (GbE) including Ginkgolic acids (a mixture of several 2-hydroxy-6-alkylbenzoic acids), terpine trilactones (ginkgolide A, B, C, J, and bilobalide) as well as flavonoid glycosides (quercetin and rutin) have been recognized (2) and benefit of GBE in the attenuation of COVID-19 based on its strong inhibitory effects on enveloped pathogenic viruses

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has been suggested (3) .

The older purposes of production and widely distribution of this freely available nutraceutical were the GBE therapeutic values in cardiovascular and ischemic diseases (4), memory loss and cognitive disorders (5) , cancer pain (6), metabolic syndrome (7) , thrombotic disorders (8) and sexual enhancement (9) but a very recent study have shown a consistent antiviral activity of Ginkgo Acids (GA) on the fusion of a wide variety of enveloped pathogenic viruses including Herpes Simplex Virus 1 (HSV-1), Human Cytomegalovirus (HCMV), and Zika virus (ZIKV) primarily through viral fusion inhibition . Furthermore, these researchers found that GA has the potential to inhibit HCMV viral DNA and HSV-1 protein synthesis by a secondary mechanism (3).In the light of recent findings on strong antiviral activities on established viral infections of permissive cells, GBE could be potentially applied to inhibit other acute viral infections (e.g. Coronavirus[(COVID-19), EBOV, ZIKV, IAV and measles)] in systemic and local pharmaceutical preparations .

Following the widespread human uses of GBE and the raising concerns on its toxicity potentials in rodents, the National Toxicology Program conducted a 2-year oral study on a sample of GbE and concluded its hepatotoxicity by clear evidence of carcinogenic activity of this extract based on an increased incidence of hepatocellular carcinoma and hepatoblastoma in mice .Although GbE is classified by the International Agency for Research on Cancer as possible human carcinogen (Group 2B) (10) , later studies didn't confirm any significant genotoxic or biologically relevant cytotoxic effects and gross gene expression changes in hepatic cells (11) . To overcome the challenges on this controversial issue on carcinogenic effects of GBE, some studies discovered the protective role of GBE low doses against basal oxidative damages and environmental toxicants e.g. Paraquate that induce oxidative DNA in in vitro and in vivo modles (12).A recent study in 2020 showed the protective role of GBE at doses of 50-150 mg/kg against the clastogenic effects of H_2O_2 , mutagenic potentials of H_2O_2 and Oxidative damage caused by H_2O_2 in liver and kidney and showed the GBE efficacy through amelioration of biochemical and oxidative stress in a dose dependent manner (13) .

Passive diffusion of a drug could be affected by its lipophilicity nature, size, molecular weight, and charge but the efficacy of available GBE pharmaceutical dosage forms remained controversial because GBE is a strong water-soluble molecule with limited tissue permeability therefore preparation of GBE Solid Lipid Nanoparticles (SLN) increases the lipophilicity of this small molecule (drug) and its diffusion across the biological barriers (14).Based on this concept we aimed in present study to provide an oral SLN formulation of GBE to improve its bioavailability and stability, increase the lipophilicity and organ(lung) entrance, postpone its rapid decomposition in body fluids and make a sustained release pharmaceutical with limited systemic toxicity .To achieve these goals, we prepared preliminary an oral GBE-SLN and evaluated its acute and repeated dose oral toxicity according to OECD 425 and OECD 407 guidelines.

MATERIAL AND METHOD

Material

Standardized GB extract (EGb761) was provided by Iran Darouk pharmaceutical Co. (Tehran, Iran) which was produced by Shaanxi Jiahe Phytochem. Co. Ltd , China and contained 24% Flavones (HPLC) and 6%Lactones (HPLC) . Cholesterol, stearic acid, glycerol monostearate (GMS), mannitol, sodium hydroxide, and potassium dihydrogen phosphate were purchased from Sigma Aldrich (Hamburg, Germany). Ethanol, acetone, and Tween 80 (polysorbate) were obtained from local branch of Merck Company.

Lab. Equipment's

Analytical Balance (OHAUS, USA), 0.02 μm syringe filter (Biomed Scientific, Sri Lanka), Digital pH Meter (SANA SL-901, Iran), Homogenizer (IKA, Germany), Ultrasonic probe (FAPAN, Iran), Freeze dryer (SBPE, Iran), Heater-Stirrer (HEIDOLPH, Germany), and Centrifuge (KOKUSAN model H-200NR) were used for preparation of the SLNs .

UV spectrophotometer (SHIMADZU, Japan), Dynamic Laser Scatter Scope (QUDIX, South Korea), Atomic Force Microscope (AFM) ,Dual scope/Raster scope C26,DME,Denmark), Scanning Electron Microscope (SEM) (HITACHI S4160, Japan), Dialysis membrane (D0405-SIGMA ALDRICH), Differential Scanning Calorimetry (DSC) (Thermal Analysis (TA), DSC Q10, Gavage syringe (Ara Teb. Fan company, Iran), and Falcons (Isola, Germany) were used for in vitro evaluation of the nanoparticles and providing the final formulation for toxicity tests. All lab devices were provided by Pharmaceutical Sciences Research

Table 1. Stepwise formulation of GbE –SLN under general [1] and stepwise optimum conditions

Formulation	Lipid (mg)			Homogenizer		Probe Sonicator	Centrifuge		Particle size (nm)	Zeta potential	PdI	Entrapment efficiency(EE)
	Chol[2]	SA[3]	GMS[4]	Time (min)	Speed (rpm)	Time (min)	Time (min)	Speed (rpm)	d 95	mV		
1st Formulations												
B1	500	-	-	10	20000	5	3	4000	150	-	-	-
B2	-	500	-	10	20000	5	3	4000	630	-	-	-
B3	-	-	500	10	20000	5	3	4000	221	-	-	-
B4	250	250	-	10	20000	5	3	4000	438	-	-	-
B5	250	-	250	10	20000	5	3	4000	298	-	-	-
B6	-	250	250	10	20000	5	3	4000	106	-	-	-
B7	150	150	150	10	20000	5	3	4000	208	-	-	-
2nd Formulations: API loading phase												
S1(B1+API)	500	-	-	10	20000	5	3	4000	105	-3.57	0.259	-
S6(B6+API)	-	250	250	10	20000	5	3	4000	69.3	2.92	0.356	-
3rd Formulations: optimized percentage of tween 80												
F1:S1(2.5% Tween 80)	500	-	-	10	20000	5	3	4000	130	-6.16	0.363	7.6%
F2 :S1(1.5% Tween 80)	-	250	250	10	20000	5	3	4000	154	-2.54	0.242	-
4th Formulations: changes in sonication time												
F2-1 (10 min sonication time)	500	-	-	10	20000	10	3	4000	95.2	-7.12	0.336	66.7%
F2-2 (2 min sonication time)	500	-	-	10	20000	2	3	4000	85.3	-8.20	0.361	68.32%
5th Formulations: change in API levels												
F2 (125 mg API)	500	-	-	10	20000	2	3	4000	85.3	-8.20	0.361	68.32%
F3(200 mg API)	500	-	-	10	20000	2	3	4000	172			65.83%
6th Formulation :Freeze dried form of F2-2												
F4 (F2+ Mannitol)	500	-	-	10	20000	2	3	4000	130	-12.4	0.393	

[1] General Optimum condition: using 1.26 g (2.5%) tween 80, 50 ml water, 12 ml ethanol, 4 ml acetone which was homogenized for 10 minutes in 20000 rpm
[2]SA: Stearic acid [3] Chol: Cholesterol [4] GMS: Glycerol mono stearate

Center at IAUTMU in Tehran.

Ginkgo Biloba Solid Lipid Nanoparticles (GBE-SLNS)

Preparation of the lipophilic phase

High shear homogenization method coupled with ultra-sonication were used to provide the desired formulation. To prepare and compare the quality of the lipid phase of 7 initial formulations, different percentages of cholesterol, stearic acid and glycerol monostearate were applied. Lipid ingredients were transferred into a mixture of ethanol and acetone respectively, heated and stirred (500 rpm, at 60-50 ° C) until the lipid phase was completely melted and disappeared in the ethanol-acetone mixture (Table 1).

Preparation of the aqueous phase:

To prepare the aqueous phase, tween 80(2.5% w/w) was added to deionized water (50 ml) on a heater stirrer to mix completely, then the lipid phase of step one was gradually added to the aqueous phase and the final mixture was homogenized (10 min, 20000 rpm) and ultra-sonicated (5 min, 70 rpm). The prepared nanoparticles (NP) were placed in ice bath (5 minutes) to form rapidly and stored at room temperature. The centrifuge method (4000rpm, 3min) was used to separate any possible NP aggregates and performed DLS analysis to select the formulations with smallest size, good particle size distribution and most stability after one hour. After initial studies on placebo preparations, EGb761 was added to the lipophilic phase of two selected formulations from above procedure and the particle size analysis, zeta potential determination and physical stability tests were performed. Optimum particle size and distribution were obtained when SLNs were prepared under following conditions: 500 mg cholesterol, 1.5% w/w tween 80, 50 ml water, 12 ml ethanol, 4 ml acetone, and 125 mg Ginkgo biloba, homogenized at 20000 rpm for 10 min, sonicated for 10 min and finally centrifuged at 4000 rpm for 3 min (Table1) .The selected formulation was characterized by Differential scanning calorimetry (DLS), as well as SEM and AFM for morphology & size evaluation .

Drug loading efficiency

As described before (Haghighi P 2018), indirect method was used to calculate the drug loading efficiency. In brief, the selected formulation (after

preparation) was centrifuged (20000 rpm for 40 min at temperature -4°C), then filtration of separated supernatant, using 0.22 µm filter syringe system, was done. Finally UV spectroscopy used to determine the drug concentration in the separated solution. The amount of drug was calculated using Equation 1 (15) .

$$LE(\%) = \frac{drug_{total} - drug_{supernatant}}{drug_{total}} \times 100 \quad (1)$$

Drug release

As described before (16) release investigation was performed using dialysis

sack method by Do405 dialysis tubing 23.15mm (Sigma, Germany).

Experimental animals and housing conditions

After getting the Ethics committee approval under the number of IR.IAU.PS.REC.1396.146 from ethics committee of IAUTMU, the study was carried out in a group of 30 mature regularly cycling female mice, aged 10 weeks, with average initial body weights of 22.86(2.74) g which were obtained from Pasture Institute of Iran. Female virgin mice were caged under standard laboratory indoor conditions, with a 12-h light/dark cycle at room temperature (23 ± 2 °C) and relative humidity (20%) with free access to tap water and a standard diet for all treatment groups. Animals had free access to standard pellet diet and drinking water ad libitum but daily food and water intakes were calculated until the end of study .The cage cleaning schedule, air filtration and recirculation, health checks and facility maintenance were carried out in accordance to the Standard Operating Procedures, and recorded. Animals were housed and maintained according to the Ministry of Health and Medical Education of Iran for the Care and Use of Laboratory Animals and CCAC Guidelines for Care and Use of Experimental Animals.

Acute and repeated dose oral toxicity study

To determine the LD50 range, the OECD 425 was implemented. For this purpose, 10 mice (5 males and 5 females, aged 8-12 weeks, weighing 20 to 24 g) were used. They were kept in the cage for 5 days to adapt to the laboratory environment. During this time, sufficient water and animal food were provided. The animals were fasted for 1–2 hour before GBE-SLN administration with free water access. The oral dose selected for the acute toxicity assessment and determination of LD50 in the animal was 2000 mg/kg, which was gavaged as a single dose. After gavage, the animals were fully observed, especially during the first 30 minutes up to 4 hours after drug administration, and their behavior was monitored. Also, they were monitored for up to 24 hours and then treated daily until day 14. In the event of death or abnormal symptoms, the information and observations of the animal were fully recorded and the mice were killed due to ethical considerations. In this study, the aim was to observe abnormal signs and mortality of animals when exposed to high doses of the drug over a short period of time.

To conduct repeated dose oral toxicity study, a total of 20 healthy female mice (5 female mice/ group) were randomly selected and divided into 4 groups (3 treatment and a vehicle control group). Treatment groups received daily doses of 0.5mg/ kg (low dose), 5mg/kg (medium dose) and 50mg/ kg (high dose) of GBE SLNs, in a volume not exceeding than 1 ml/100 g B.W./mice, for 7 days a week administrations. Animals from control group received water by gavage in the same volume, which was used in treatment group.

The general behavior of the animals was observed daily and before any daily administrations, the female mice were weighed at the same routine daily time. Water intake, food consumption, and body weight were measured once a day. According to OECD 407 toxicity assessment guideline (OECD Guidelines, TG 407, 2008), total body weights, organ weights, macroscopic organ evaluations, hematology, serum biochemistry and organ histopathology were assessed after performing the 28 day study. At day 29th, all mice in both groups were anesthetized for blood collections by heart puncture under light carbon dioxide anesthesia. One part of their whole blood was kept in acid-washed cryo-tubes at -80 °C, while the remaining part of blood was centrifuged at 3000×g/10 min in a refrigerated centrifuge at 4 °C to separate the serum, and then stored at −80 °C until biochemical analyses.

Histopathological examinations:

During necropsy study, all necessary organs including liver, kidney, spleen, heart, lung, brain, uterus and ovaries were dissected out. The whole organs weighted and fixed in formaldehyde 10% for 24 h and then embedded in paraffin blocks, sliced into 5-µm sections and stained with hematoxylin-

Table 2. GbE release percentage at different time intervals (n=3)

Time(h)	Before Freeze drying		After Freeze drying	
	Mean %(SD)	SD	Mean %	SD
0.33(20min)	7.51(0.715)	0.715	12.73	2.3
0.66(40min)	11.08	2.290	16.4	4.1
1	14.75	2.285	23.37	2.9
2	22.97	3.125	33.47	3.1
4	35.61	3.960	44.16	2.8
6	45.83	1.916	56	2.4
24	63.59	5.220	68	4.3
48	73.94	3.920	72	3.1
72	85.28	2.786	83.78	2.21
76	82.96	1.595	82.15	1.4

eosin (H&E) for histopathological evaluation. The sections were examined under the light microscope (Olympus BX-51; Olympus, Tokyo, Japan) by expert animal pathologist and scored.

Statistical analysis

In this analysis, treatment and control groups were compared with each other. When variances were not significantly different, data were analyzed by one-way analysis of variance (ANOVA) and the Student's t-test. A stepwise multiple comparisons procedure was used to identify the sample means that were significantly different from each other. We used post hoc test whenever a significant difference between three or more sample means has been revealed by an analysis of variance(Anova) .Values were expressed as means ± SD. The level of significance was set at $p < 0.05$. All statistical methods were performed by SPSS version 21.

RESULTS

GBE-SLN STEPWISE FORMULATION AND CHARACTERIZATION

Step 1: After preparing the first 7 initial Nano formulations and based on particle size analysis, formulations B1 and B6 were selected as the optimum carriers for the rest of manipulation in phase 2.

Step 2: The basic formulations were loaded by GbE (API) and the secondary formulations (S1 and S6) were considered as the subjects for the next zeta potential and polydispersity index (PDI) analysis. In this step, S6 was removed from next manipulation due to its higher zeta potentials (lower stability) compared to S1 (2.92 *vs.* -3.57 mV) despite of its smaller particle size (69.3 *vs.* 105 nm).

Step 3: In third step of formulation, the percentage of tween 80 was reduced from 2.5% w/w/ to 1.5% w/w which made a more stable formulation(F2) with optimum condition (Zeta potential: -7.12mV, PDI: 0.336, and particle size: 95.2 nm).

Step 4: At the next phase (phase 4),the sonication time was decreased from 10 min to 2 min, and this manipulation causes smaller particles with increased stability (F2: Zeta potential : -8.20mV, PDI: 0.361, and particle size: 85.3nm).

Step 5: At the last stage of above procedure, we tried to load more API to F2 (200 mg instead of 125 mg) but increased API level made larger sized particles (85 increased to 172nm) in F3 formulation. Based on these stepwise formulations, we used freeze-drying method to stabilize the optimum formulation (F2) (Table 1).

GBE-SLN Release Profile

According to optimum entrapment efficiency for F2 preparation, this formulation was used for release profile studies of GbE which showed us the optimum entrapment efficiency. To achieve this goal and after preparing the F2 SLN sample , nanoparticles of preparation F2 was transferred into dialysis membrane and sampling was done at specific time intervals and this experiment repeated 3 times. According to the obtained data (Table 2), the peak of GbE release was observed within 72 hrs. Afterwards, the rate of the API release declined and reached to a constant level (Fig. 1). The cumulative release percentage of GbE after freeze-drying is explained in Table 2.

GBE-SLN Morphology

We used SEM (Fig. 1) and AFM (Fig.2) analysis to evaluate the morphology of optimized formulation in fresh suspension dosage form before and after freeze drying (F2 *vs.* F4).The particle size

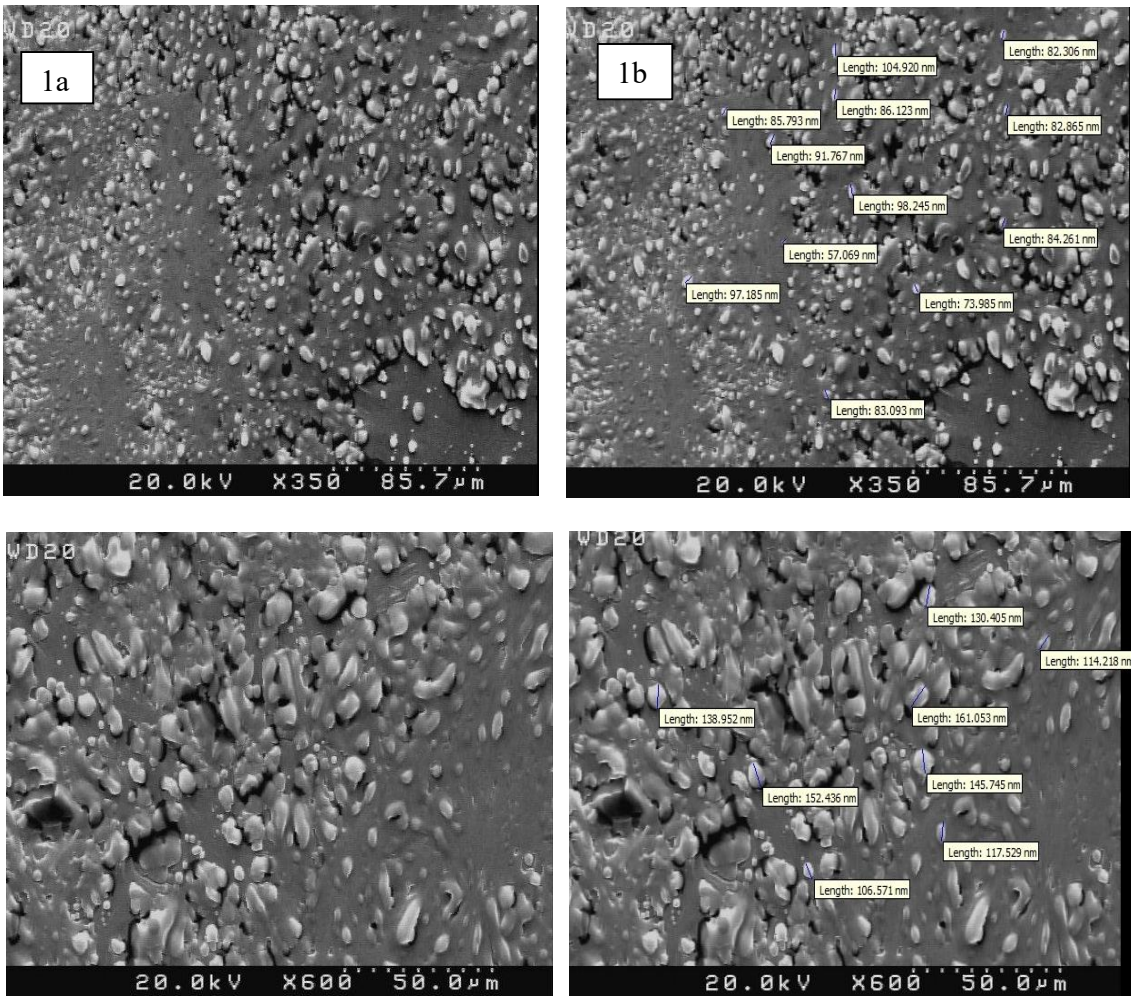

Fig. 1. The SEM images optimized GbE –SLN before and after freeze-drying: Fig 1a and 2a show F2 formulation before freeze-drying, Fig 1c and 1d show F4 formulation after freeze-drying

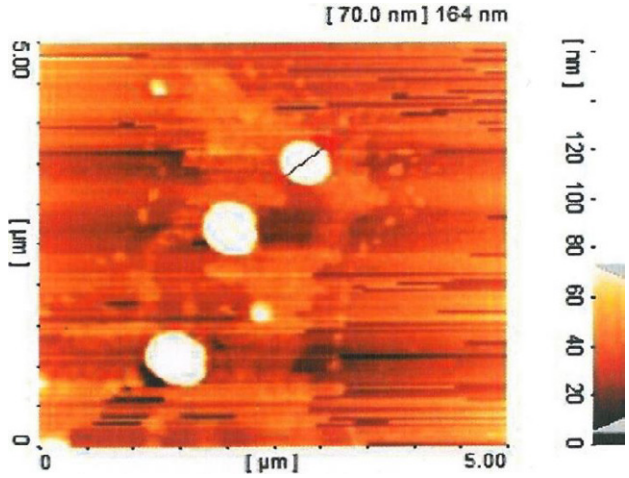

Fig. 2. AFM image of GBE-SLN fresh suspension before freeze dry (F2 formulation)

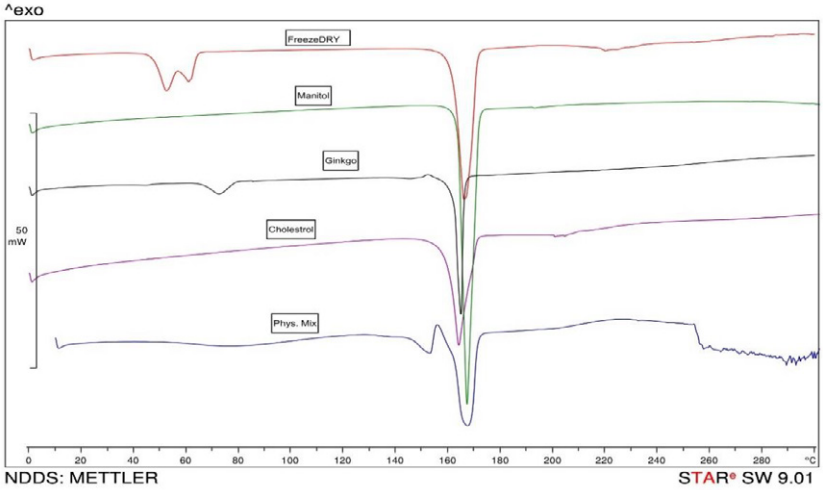

Fig. 3. Differential scanning calorimetry of GBE-SLN

analysis which have shown in Figs. 1 and 2 were in close agreement with the DLS results (Table 1). In Fig. 1c and 1d, which corresponds to the image of the freeze-dried sample, particle size is larger and the particles homogeneity is also reduced.

GBE-SLN Differential scanning calorimetry analysis

The DSC thermograms indicate the enthalpy changes and the specific heat capacity of the materials in the final formulation. The peak of each compound can be seen in Fig. 3.The peak of GbE was obtained at 72.56° C and 163.64° C, cholesterol at 163.27 ° C and 204.91° C, mannitol at 164.38° C. When the three substances were combined, a peak was seen at 166.5 ° C and with the freeze-dried formulation, a peak was seen at 165.7 ° C. Due to the structure of the components in the GbE and cholesterol, the probability of the formation of hydrogen bonds confirms the slow release profile of the final formulation in this study.

GBE-SLN Acute oral toxicity

The result suggested that the LD_{50} of GBE-SLN is greater than 2000 mg/kg body weight. There was no signs of toxicity or mortality, no significant change in body weight, organ weight, tissue macroscopic shapes and colors, behavior, renal function test, liver function test and lipid profile after 24 hour from single dose administration as well as 14 days from 2000mg/kg of GBE-SLN oral administration in both genders.

GBE-SLN Repeated Dose Oral Toxicity

Clinical

No mortality was observed during this 28 days study in female mice or any sign of usual overdose complications in daily oral doses up of 50 mg/kg. All animals appeared normal without any observable/recordable clinical change in their total body weights, food and water consumption. Feed consumption was not reduced in rats treated with GBE-SLN from 1st to 4th week of study, but the mean total body weight in high dose (50 mg/kg /day) of GBE-SLN treated was significantly increased compared to vehicle control group (36.53(1.92) *vs.* 34.26(1.25)g, p=0.023) (Table 3).

Necropsy

No abnormal change was observed grossly in mice treated with GBE-SLN doses and vehicle control after 28 days of study .Except the mean weight of liver in high dose GBE-SLN treated mice which was significantly reduced compared to vehicle control group [1.44(0.10*) v.s* 1.48(0.18g), p=0.027]. The mean weights of other organs including heart, brain, kidneys, liver, lungs, uterine, ovaries and spleen in 3 different doses remained unchanged compared to vehicle control group (Table 3).

Hematology

Abnormal hematological changes was detected in high dose GBE-SLN treated mice .MCV level in

Table 3. Mean (SD) of total body weights at Day 1, 14, 28 and organ weights at day 29 of GBE-SLNs oral administration compared to vehicle control group

Variables	[A] Low dose GBE-SLN (0.5 mg/kg, n=5)	[B] Medium dose GBE-SLN (5 mg/kg, n=5)	[C] High dose GBE-SLN (50 mg/kg, n=5)	Vehicle Control (n=5)	P-value
Total Body Weight changes					
Weight at day 1	22.13(1.76)	22.88(1.17)	22.94(2.12)	22.78(5.49)	NS
Weight at day 14	32.11(2.59)	32.48(1.55)	32.29(2.40)	32.23(2.38)	NS
Weight at day 28	34.14(1.65)	34.89(1.69)	36.53(1.92)	34.26(1.25)	0.023*[C]
Organ weights at day 29					
Heart	0.16(0.12)	0.15(0.87)	0.17(0.01)	0.15(0.02)	NS
Brain	0.39(0.13)	0.40(0.05)	0.38(0.026)	0.40(0.03)	NS
Kidney	0.37(0.08)	0.39(0.07)	0.34(0.04)	0.40(0.05)	NS
Liver	1.48(0.18)	1.48(0.18)	1.44(0.10)	1.48(0.18)	0.027*[C]
Lungs	0.31(0.04)	0.33(0.09)	0.32(0.06)	0.30(0.03)	NS
Uterine & varies	0.191(0.03)	0.188(0.09)	0.180(0.04)	0.19(0.04)	NS

Table 4. Hematological parameters of female mice treated with GBE-SLN for 4 weeks at day 29 .

Variables	[A] Low dose GBE-SLN (0.5 mg/kg, n=5)	[B] Medium dose GBE-SLN (5 mg/kg, n=5)	[C] High dose GBE-SLN (50 mg/kg, n=5)	Vehicle Control (n=5)	P-value
WBC($10^3/\mu$L)	7.16(5.27)	7.15(3.27)	5.53(3.51)	7.11(5.57)	NS
RBC($10^6/\mu$L)	10.33(0.45)	10.61(0.75)	10.56(0.58)	10.91(0.35)	NS
Hb(g/dL)	15.37(0.24)	15.19(0.59)	15.25(1.03)	15.43(0.54)	NS
HCT (%)	57.16(1.32)	56.16(1.12)	58.04(3.86)	56.86(1.82)	NS
MCV(fL)	52.95(1.87)	52.55(1.48)	54.93(2.12)	52.15(1.57)	NS
MCH(pg)	14.95(0.34)	14.44(0.19)	14.44(0.51)	14.15(0.74)	NS
MCHC(g/dL)	27.95(1.41)	26.94(1.81)	26.28(1.04)	27.15(1.21)	NS
PLT($10^3/\mu$L)	886.83(159.38)	851.83(128.65)	1041.90(196.73)	831.83(147.85)	0.041*[C]

Table 5. Serum biochemical parameters of female mice treated with GBE-SLN for 4 weeks.

Variables	[A] Low dose GBE-SLN (0.5 mg/kg, n=5)	[B] Medium dose GBE-SLN (5 mg/kg, n=5)	[C] High dose GBE-SLN (50 mg/kg, n=5)	Vehicle Control (n=5)	P-value
Glucose(mg/dl)	95.66(13.04)	125.80(95.15)	74.66(65.04)	145.80(95.15)	NS
Creatinine	1.08(0.10)	1.32(0.03)	1.18(0.41)	1.07(0.05)	NS
Triglyceride(mg/dl)	101(5.07)	102(13.26)	119.33(13.05)	97(17.56)	NS
Cholesterol(mg/dl)	104.26(11.31)	108(7.97)	109.66(11.01)	109(9.27)	NS
HDL(mg/dl)	65.83(2.16)	67(1.64)	61.33(4.16)	67(4.24)	NS
LDL(mg/dl)	16(1.36)	17.70(3.36)	17(3.46)	14.80(2.16)	NS
AST(U/L)	201.56(78.83)	168(52.26)	213.66(78.83)	168(52.26)	NS
ALT(U/L)	66.56(13.01)	59.20(10.08)	62.66(15.01)	58.20(10.08)	NS
Alkaline phosphatase(U/L)	3589.23(31.5)	364(41.61)	379.33(32.34)	344(44.81)	NS
Calcium(mg/dl)	10.13(0.79)	10.59(0.60)	10.53(0.49)	10.68(0.80)	NS

treatment group increased significantly compared to vehicle control [54.93(2.12) *v.s* 52.15(1.57) fL, p=0.015]. Other than MCV, PLT was significantly increased in GBE-SLN treated mice compared to vehicle control group [1041.90(196.73) *v.s* 831.83(147.85), *p*=0.041].Other blood factors remained unchanged in high dose treatment group at day 28 (Table 4). All blood factors remained normal in low and medium doses at day 29 of this repeated dose oral toxicity study.

Serum Biochemistry

As described in Table 5, Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and all other biochemical toxicity related factors remained unchanged in all three GBE-SLN treated groups of mice compared to vehicle control group (p<0.05). Lipid profile of treated all animals remained also unchanged (Table 5).

Histopathology

Spleen

Microscopic evaluation showed normal feature in spleen sections of GBE-SLN groups (Fig. 4A1, 4A2) and vehicle control group.

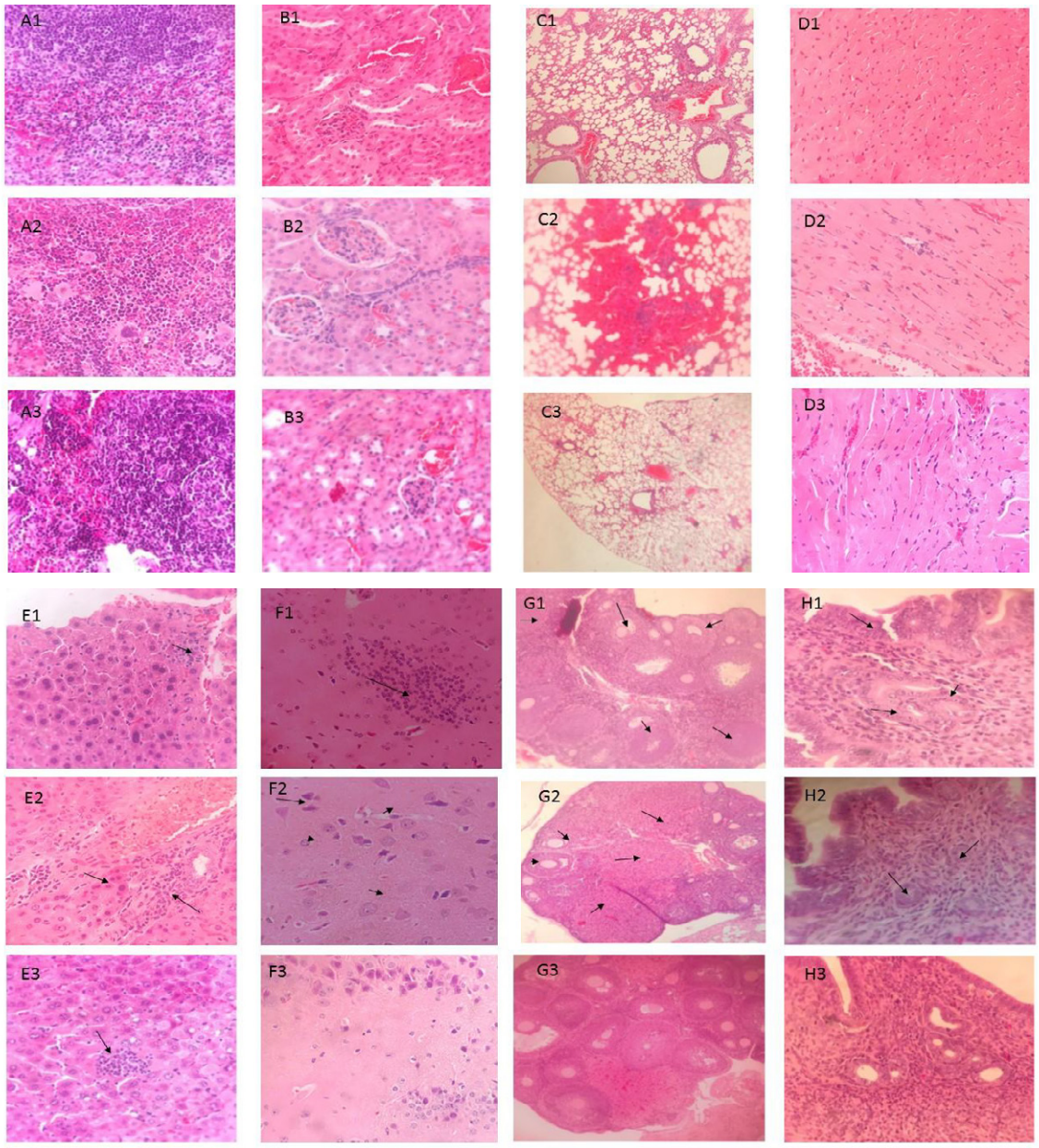

Fig. 4: Histopathological effects of GBE-SLN different doses in spleen, kidney, lung, heart, liver, brain, and ovary and uterine of female mice compared with vehicle control group. Photomicrographs of all sections stained with hematoxylin and eosin and showed in the same magnifications(x100): A1: Normal splenic tissue (GBE-SLN), A2: Splenic tissue with prominent megakaryocytes (GBE-SLN), A3: Normal splenic tissue (Vehicle control), B1: Normal renal tissue (GBE-SLN), B2: Mild congestion in renal tissue (GBE-SLN), B3: Normal renal tissue (Vehicle control), C1: Normal pulmonary tissue (GBE-SLN), C2: Focal Hemorrhage in pulmonary tissue (GBE-SLN 50 mg/kg/day), C3: Normal pulmonary tissue (Vehicle control), D1: Normal cardiac tissue (GBE-SLN), D2: Cardiac tissue with mild congestion (GBE-SLN 50 mg/kg/day), D 3: Normal cardiac tissue (Vehicle control), E1: Hepatic tissue with mild vacuolar degeneration. There was focal infiltration of mononuclear cells in hepatic tissue (arrow) (GBE-SLN, 50 mg/kg/day), E2: Periportal infiltration of mononuclear cells and hepatocyte necrosis (GBE-SLN, 50 mg/kg/day), E3: Hepatic tissue with extramedullary hematopoiesis (GBE-SLN50 ,mg/kg/day), F1: Cerebral tissue with focal gliosis (GBE-SLN), F2: Cerebral tissue with schematic changes in cellular morphology in cortex layer (GBE-SLN), F3: Cerebral tissue with normal histology (Vehicle control), G1: Ovarian tissue with many corpus luteum and many antral follicles (GBE-SLN), G2: ovarian tissue with prominent corpus luteum and pre-antral follicles (GBE-SLN), G3: Normal ovarian tissue with many follicles in different stage (Vehicle control), H1: Uterine horn layers with normal histology. Endometrial glands was in proliferative phase (GBE-SLN), H2: Uterine horn with normal histology. Endometrial gland was in proliferative phase (GBE-SLN), H3: Normal endometrial tissue (Vehicle control)

Kidney

Kidney sections of two third of female mice in GBE-SLN group showed normal feature (Fig. 4B1) but mild congestion was seen in kidney of one mice in high dose (50mg/kg/day) treated group (Fig.4B2) compared to the vehicle control group (Fig. 4B3).

Lung

Despite normal feature in lung sections of most of female mice in GBE-SLN group (Fig. 4C1), one mice in high dose group showed focal Hemorrhage (Fig. 4C2) compared to normal feature in vehicle control group (Fig. 4C3).

Heart

Heart sections of most of female mice in GBE-SLN group showed normal feature (Fig. 4D1) but mild congestion was seen in the heart of few cases of high dose group of animals (Fig. 4D2) compared with vehicle control group (Fig. 4D3).

Liver

Although any fatty change was not detectable in liver sections of animal , high dose(50mg/kg/day) GBE-SLN animals showed mild vacuolar degeneration and focal infiltration of mononuclear cells (Fig. 4E1), periportal infiltration of mononuclear cells and hepatocyte necrosis (Fig. 4E2) and extramedullary hematopoiesis (Fig. 4E3) compared to normal liver appearance in medium dose(5mg/kg/day) ,low dose(0.5 mg/kg/day) and control group.

Brain

Few dissected brains of female mice in GBE-SLN group showed focal gliosis in cerebral tissues (Fig. 4F1). Schematic changes in cellular morphology in cortex layer of cases(Fig.4F2) was also the second abnormal feature in GbE exposed animals compared with normal cerebral feature in medium dose(5mg/kg/day) ,low dose(0.5 mg/kg/day) and control group (Fig. 4F3) .

Ovary

Ovaries of all female mice in high dose GBE-SLN group showed corpus luteum and antral follicles (Fig. 4G1) and pre-antral follicles (Fig. 4G2) while medium dose (5mg/kg/day) ,low dose(0.5 mg/kg/day) and the control group showed normal ovarian tissue with many follicles in different stages (Fig.4G3).

Uterine

All treated doses and control groups showed normal uterine in microscopic evaluations (Fig. 4H1, 4H2, 4H3).

DISCUSSION

Solid lipid nanoparticles (SLNs) are new colloidal drug carriers with biocompatible lipid nanostructures compared to the polymeric or inorganic nanoparticles for controlling the rate of drug release and improving the lasting effects of any pharmaceutical molecule (Solmaz Ghaffari 2011). One of the other advantages of SLNs compared to other Novel drug delivery systems (NDDS) is their lipid nature and small particle size which enable them to penetrate from biological barriers even without necessary functionalization . These specifications have made SLNs as one of the most attractive representatives of lipid-based nanosystems with great potentials for targeted drug delivery in different diseases (Christos Tapeinos 2017) .Based on this concept, we decided in this study to provide GbE –SLNs for future application in Covid 19 and evaluate the safety of GBE-SLN in acute and repeated dose oral toxicity models .To the best of our knowledge very few herb based SLNs have been clinically developed for commercial applications because of insufficient loading capacity, lack of efficacy and unknown toxicity .

Before toxicity assessment, we designed the smallest nanoparticles in size with optimum PDI by modifying the type and level of lipids, level of surfactant, time and speed of homogenization, time of sonication as well as ratio of drug to lipid . We followed the effects of any mentioned variables(alone and in combination) on particle size, PDI and zeta potential .In the next step, high shear homogenization technique was selected to improve the loading capacity of optimized lipid carriers by adjusting several factors in a stepwise formulation. Based on our first experience in providing GBE-SLN (Haghighi et al 2018), we justified many independent variables to improve the dependent variables and in final formulation, the ratio of ethanol/acetone, the ratio of active agent /lipid, the homogenization and sonication condition as well as the percentage of surfactant were totally different. In parallel to successful efforts and exciting achievements in formulating a sustained release GBE-SLN with optimum loading capacity

with the release rate of 7.51% in first 20 minutes with capacities to reach to the peak release (85.28%) in 72 hours, drug release results have indicated the probability of independency of release profile to the particle size and other investigated variables. The drug release profile of the SLNs of GbE in this study was similar to our previous study, so it seems that the type of lipid as carrier which can interact with the API, is the main effective variable on drug release profile, although for fully confirm of this hypothesis, more studies should be designed in next scale up studies .

In the second part of this study, we assessed the acute and repeated dose oral toxicity of GBE-SLN in mice model in three different dose levels (0.5.5. 50mg/kg) because the oral toxicity of GBE-SLN could be different from regular GBE based of pharmacokinetic changes and different biodistribution. Acute toxicity studies on the standardized GB extract (EGb761) showed LD50 values of 7700 mg/kg (ppm) in mice by oral route (17)which is in accordance with our acute oral toxicity of GBE-SLN without any sign of toxicity or mortality in doses up to 2000mg/kg according to OECD 425 guideline in both genders of mice .

In repeated dose oral test despite aurvival of all 20 mice in 4 dose groups , the mean weight of animals in high dose group was slightly increased but the major findings regarding the oral toxicity of GBE-SLN in 28 days female mice model(OECD 407) , including clinical signs and symtpoms ,water and food consumpton as well as necropsy and biochemical records didn't show any abnormality which is in accodrance with a recent study on similar doses (50mg/kg/day) of GbE (13). Although in the toxicity assessment of GbE,former studies showed varied degrees of effect on liver, thyroid, and nose of GbE exposed animals which were consistent across their sex, species, and exposure period (18). We didn't consider GBE-SLN toxic potentials in nose and thyroid of our study subjects but decreased liver weights (p=0.027*,Table 3) ,mild vacuolar degeneration ,focal infiltration of mononuclear cells , hepatocyte necrosis and extramedullary hematopoiesis in the liver sections of all GBE-SLN female mice (Fig 4E1-3) are trustable signs for dose depentent hepatotoxicity that prove the persistence of the hepatotoxic effects of Ginkgo biloba leaf extract even in a Nano formulation. Fortunately lower doses of GBE-SLN (0.5 and 5 mg/kg/day) eliminated the risk of hepatotoxicity in treated rat which could be considered in next studies on its antiviral properties .

Mild congestions in the tissue sections of heart and kidney of animals were the other GBE-SLN induced organ toxicities which were observed in high dose group of female mice (Fig 4 B2 and D2) .These observations were accompanied by some hematological disorders which explained in Table 4 .We found mild primary thrombocytosis and increased MCV levels in GBE-SLN group of mice [1041.9010^3/μL (196.73) PLT vs. 831.8310^3/μL (147.85), p=0.041].Clotting is a natural protection against any type of bleeding and we found at the same time pulmonary hemorrage in the high dose GBE-SLN treated mice after 28 days of oral administration. The incidence of respiratory toxicity of GbE by inducing transitional epithelium and respiratory epithelium hyperplasia as well as atrophy, metaplasia, nerve atrophy, and pigmentation in the olfactory epithelium in rats were previously described (19) which was not detected in our experiment by GBE-SLN but we described possible hemorrhagic effects of GbE by concommitant hematological and pulmonary disorders . These occasional observations in doses upper than 50 mg/kg/dar can not support the benefits of GBE-SLN for thrombosis as an effective inhibitor of thrombin which is recently suggested for Biflavones from Ginkgo biloba (8),but this is likely to be the case in future studies in lower doses .Other histopathological changes such as heart congestion, kidney congestion, cerebral gliosis , abnormal cellular changes in cerebra and lack of narmal ovarian follicular development which we mentioned in Fig 4 are other possible dose dependent toxic responses of Gb-SLN which we observed with doses of 50mg/kg/day in few cases of present study .This wide range of Gb-SLN induced organ toxicities reflects the total biodistribution of GBE-SLNs but we recorded the total safe of Gb-SLN in lower oral doses(0.5-5mg/kg) at the same setting in present experiment .

Many studies have shown the inhibitory role of GbE components mainly flavonoids, terpene lactones, and Ginkgo acids on vascular angiogenesis (20) a distinguished factor of pulmonary pathobiology of Covid-19 from that of equally severe influenza virus infection (21) . GBE antagonize the platelet activating factor (PAF) (22) which has critical role in pregression of viral infections e.g HIV (23) moreover GBE has strong antioxidant effecst (24) which is another effecitve therapeutic strategy agaist Covid -19 crisis (25).GBE regulats the release

of excitatory amino acids (26) and reducing the interleukin-6 cytokine levels in human studies (27) which have excellent prognsotic values for Covid-19 clinical signs and symptoms (28)

Based on our experineces in general safety and systemic tolerance to GBE-SLN doses up to 5 mg/kg/day in current animal study we highly recommend to evaluate the effecacy of present dosage form against Covid-19 critical crisis through human studeis . GBE-SLNs may exert simillar protective effects against free radicals toxicities (13) and brain disorders (29) in lower doses without adverse health effects which was previously described preclinical and clinical settings with minum adverse health effects by regular dosages (9) moreover the inhalation rout of adminstration of Gb-SLNs could be considered as another effective strategy againt Covid -19 with necessary clinical evaluations .

Our selected Nano formulation from Ginkgo Biloba didn't show any sign of toxicity in acute and in repeated dose 28 day study in doses up to 5 mg/kg/day which was emphasized clinical ,hematological ,biochemical ,necropsy and histopathological which suggest it as a promising supplement for Cocid-19 induced systemic and respiratory symptoms. Considering the histopathological observations and mild toxic responses in the liver of animals as well as partial toxic effects in brain ,renal ,heart and ovaries of exposed mice we consider 5mg/kg/day as Non Observed Adverse Effect Level(LOAEL) of GE-SLN and we suggest further Randomized Clinical Trials on this Nano formulation as a new safe strategy against Covid-19 and other viral infections based on mechanistic evaluations of GB and pathogenic features of Covid-19.

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CONFLICT OF INTEREST

Authors declare no conflict of interest in present work.

LIST OF ABBREVIATIONS

AFM Atomic Force Microscopy
API Active Pharmaceutical Ingredient
CCAC Canadian Council of Animal Care
DLS Dynamic Light Scattering
GBE Ginkgo Biloba Leaf Extract
GBE-SLN Ginkgo Biloba Leaf Extract –Solid Lipid Nanoparticle
H&E Hematoxylin-Eosin
IAUTMU Islamic Azad University, Tehran Medical Sciences University
LD50 ethal Dose in 50% of Population
NDDS Novel drug delivery systems
NOAEL No Observed Adverse Effect Level
NP Nanoparticle
OECD The Organization for Economic Co-operation and Development
SLN(s) Solid-Lipid Nanoparticle(s)
SEM Scanning Electron Microscopy
TG Test Guideline

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