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ACUTE TOXICITY STUDIES OF NANO-FORMULATIONS OF *GLYCYRRHIZA GLABRA* EXTRACT IN SWISS ALBINO MICE

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ABSTRACT

Licorice (*Glycyrrhiza glabra*) has been used as a medicinal herb in various systems of medicine owing to its widespread biological activities. The biological activities may be attributed to polyphenols present in licorice. Since the polyphenols have been reported to have poor bioavailability, it was decided to formulate bioactive licorice extract into suitable nano-formulations viz. polymeric nanoparticles and liposomes. The aim of the current study is to evaluate the acute toxicity of these nano-formulations of licorice. In this study, Swiss Albino mice were given a single dose of nanoparticles and liposomes at 5000 mg/kg and observed for two weeks after administration. During this period, the animals were observed for any abnormal behavior, mortality, body weight changes, any other signs of toxicity and food consumption. Detailed hematological, biochemical and histopathological evaluation of organs was performed for the animals of each group. The Mn-GNP as well as LDPI formulations were well tolerated and no signs or symptoms of toxicity were observed. No significant difference in the hematological and biochemical parameters were seen in any group. The histopathological evaluation of the organs of animals of test group was comparable to that of control group. No mortalities were observed during the study period. The LD_{50} of both the formulations was more than 5000 mg/kg indicating that they are safe.

KEYWORDS: Acute toxicity, nanoparticles, liposomes, *Glycyrrhiza glabra*, Swiss Albino mice, licorice.

INTRODUCTION

Drugs from plant origin have been used for the treatment of various diseases as well as ailments for generations. About 80 % of the world's population relies on medicines from natural sources, primarily plants.[1] Though herbal drugs have been regarded as relatively safe, there have been incidences in the past of adverse reactions such as allergy, toxic effects, etc. after administration of herbal drugs.[2] Thus there is an urgent need for generating data on safety assessment of drugs of herbal origin.

Licorice is one such herb known to mankind since ages. Due to a wide array of biological activities exhibited by the herb, it has been used in various systems of medicines. The various biological activities of licorice constituents which are reported include anti-depressant, memory enhancing, anti-obesity, ulcer healing, anti-microbial, anti-viral and anti-mycobacterial.[3] The biological activities of licorice are mainly attributed to the presence of polyphenols. These phenolic compounds have been reported to have poor oral bioavailability.[4] It is thus necessary to formulate the bioactive licorice extract (containing polyphenols) into suitable dosage form so that its potential as a medicine is well explored. In view of this, two nano-formulations were prepared by incorporating licorice extract – Polymeric nanoparticles and Dry Powder for inhalation liposomes (LDPI). Polymeric nanoparticles containing licorice extract were prepared using gelatin by double desolvation technique and LDPI were prepared by freeze drying the liposomes of licorice extract formulated using soyabean phosphatodylcholine and cholesterol.

The risk and safety assessment of licorice as well as licorice extract is reported. There is also significant data on the toxicity profile of licorice.[5] But there are no reports on the toxicity of the nanoparticle as well as liposome formulations containing licorice extract. In order to ascertain the safety and tolerability of these nano-formulations, the present study was undertaken and the acute toxicity of the nano-formulations prepared using *Glycyrrhiza glabra* extract were evaluated in Swiss Albino mice.

MATERIALS AND METHODS

Materials

Mannosylated gelatin nanoparticles (Mn-GNP) and LDPI formulations were obtained from Department of Pharmacognosy and Phytochemistry, Bombay College of Pharmacy, Mumbai.

Preparation of nanoparticle formulation of licorice extract[6]

The nanoparticles were prepared using gelatin by double desolvation technique. Briefly, required quantity of gelatin (type A, 175 bloom, Sigma) was dissolved in distilled water with the aid of heat. The first desolvation of gelatin was achieved by addition of acetone. The desolvated high molecular weight gelatin was precipitated and was separated by centrifugation. The separated gelatin was re-dissolved in water and the pH of the solution was adjusted to 2.5. Second desolvation was done by drop wise addition of licorice extract (LE) solution in acetone to the desolvated gelatin under stirring at 500 rpm. The formulation comprised of LE and gelatin in a ratio of 1:6 by weight. The system was kept under stirring for 12 – 16 hours till complete evaporation of acetone was achieved. The primary nanoparticles thus formed were conjugated with mannose. The mannosylated gelatin nanoparticles (Mn-GNP) were then freeze dried and reconstituted in sterile water before administration to animals.

Preparation of LDPI containing LE

Formulation of liposomes of LE was carried out by lipid thin film hydration method[7] using soyabean phosphatidylcholine (Lipoid S100) and cholesterol. Lipid thin film was prepared in rotary vacuum evaporator (Rotavapor® 210 - Buchi, Flawil, Switzerland) using cholesterol and soyabean phosphatidylcholine with LE incorporated in it. LE and lipids were used in a ratio of 1:9 wherein the lipid part comprised of 35 parts of cholesterol and 65 parts of soyabean phosphatidylcholine by weight. Lipid film was hydrated using filtered distilled water by agitation for 5 minutes. This was then sonicated using bath sonicator to obtain liposome suspension. Trehalose, in an amount of 4 times the liposomal solid content by weight, was mixed with the liposome suspension and freeze dried to obtain dry powder. The porous cake formed was passed successively through #200 and #240 mesh to obtain liposomal dry powder for inhalation (LDPI).

Experimental animals

Six to eight weeks old Swiss Albino mice of either sex weighing between 18 - 25 g were obtained from Central Animal House, Bombay Veterinary College, Mumbai, India. The

animals were kept in Independent Ventilated Cage (Model IVC-II, Suzhou Fengshi Laboratory Animal Equipment Co. Ltd., China) with rice husk bedding. Individual animal was identified by specific marking and cages were identified with label pasted on cages with relevant information. Animals were housed at a temperature of 23 ± 2°C and relative humidity of 50 ± 10 %. A 12 hour light and 12 hour dark cycle was followed. All animals had unlimited free access to water and standard pelleted laboratory animal diet. The animals were acclimatized for 7 days before initiation of the experiment. The experiment protocol was approved by Institutional Animal Ethics Committee, Bombay Veterinary College, Parel, Mumbai, India (No.MVC/IAEC 21/2014 dated 02.06.2014). The grouping of the animals is shown in table 1.

Table 1: Grouping of animals used in the study.

Group	Formulation/ Sample administered	Route of administration	No. of animals
A	Vehicle (sterile water for injection)	Intravenous	6
B	Mn-GNP formulation	Intravenous	6
C	Blank liposome formulation	Inhalation	6
D	LDPI formulation	Inhalation	6

Dose administration

The Mn-GNP were administered to the animals intravenously by tail vein technique. The LDPI formulation as administered by inhalation route using a calibrated nose-only apparatus specially designed for administration of dry powder inhalation formulations to mice.[8] Corresponding control groups (group A for intravenous route and group C for inhalation route) were also administered the vehicle by the designated route.

Acute toxicity study

For the acute toxicity study, animals were administered formulations at a dose of 5000 mg/kg. Individual animals were observed for any signs of toxicity once in the first 30 minutes after administration and daily thereafter for 14 days. Daily cage side observations included: presence of tremors, convulsions, salivation, diarrhea, lethargy, sleep, food consumption and coma. Animals were also observed for changes in skin, fur, mucus membrane, eyes, somatomotor activity, respiratory activity and behavior pattern. Weights of the animals were recorded before administration and at least weekly thereafter. At the end of 14 days, surviving animals were humanely euthanized and LD_{50} was determined.[9]

Hematological and Biochemical parameters

Blood samples were collected from all animals after terminal sacrifice into heparinized tubes. Following hematological and biochemical parameters were estimated: hemoglobin (Hb), red blod cospuscles (RBC), white blood corpuscles (WBC), platelets (PLT), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), neutrophils (N), eosinophils (E), lymphocytes (L), monocytes (M), aspartate triaminase (SGOT), alanine transaminase (SGPT), alkaline phosphatase (ALP), Total proteins (TP), albumin (Alb), globulin (Glb), blood urea nitrogen (BUN), creatinine (Creat). Hematological parameters were measured using Autoanalyzer (Vector Series BC-1800, Mindray, China) whereas biochemical parameters were estimated using a Fully Automatic Batch Analyzer (Sphera, Edif Instruments, Italy). The hematological and biochemical parameters of the animals which received formulations were compared with those of the control group.

Histopathology

For the animals which received Mn-GNP formulation, the following organs were isolated: brain, heart, liver, kidney and lungs. For animals which received LDPI formulation, lungs were isolated. All the organs were stored in 10% buffered formalin for 48 hours. The organs were then embedded in paraffin wax, sectioned at 5 micrometers and observed for histopathological changes after staining with hematoxylin and eosin. The histopathology of the organs of the animals which received formulations were compared to those of the control group.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, Inc.) software. All data are shown as mean ± standard deviation. Comparison of data of control and treatment groups was done by Student’s t-test at 95% confidence interval.

RESULTS AND DISCUSSION

Preparation of formulations

Both the formulations were suitably evaluated *in vitro* and found to possess satisfactory physic-chemical properties (unpublished data). The particle size of the formulations was in the nano range (200 – 300 nm) with good drug loading. After satisfactory evaluation, they were taken ahead for *in vivo* studies.

Acute toxicity study

OECD guideline specifies a limit test dose of 5000 mg/kg for acute toxicity testing of formulations. There were no signs of toxicity or any other adverse effects on animals which received the formulations at a dose of 5000 mg/kg. The Mn-GNP and LDPI formulations of *Glycyrrhiza glabra* extract thus have an LD_{50} value of more than 5000 mg/kg as none of the animals died during the experiment period. The formulations thus can be assigned class 5 status (LD_{50} > 5000 mg/kg) according to the chemical labelling and classification of acute systemic toxicity recommended by OECD, which was the lowest toxicity class. Any substance with an LD_{50} between 5000 and 15,000 mg/kg is considered nontoxic.[10] The effect on body weight as well as organ weight of the animals which received the formulations as well as the control group animals are summarized in figures 1 and 2 respectively.

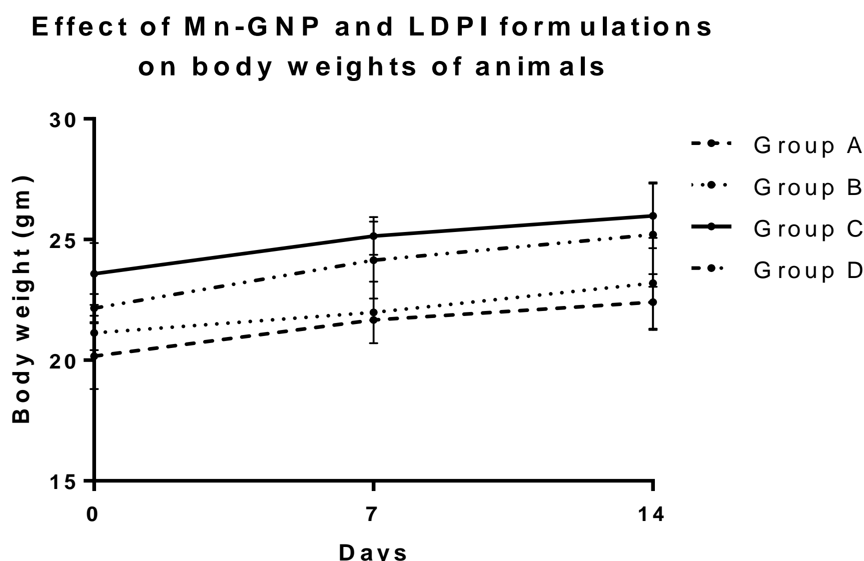

Fig. 1: Effect of Mn-GNP and LDPI formulations on body weights of animals. Values reported are Mean ± SD, n = 6.

As evident from the body weight data of animals, there is no marked difference in the body weights of the animals during the study period. None of the groups of animals showed any abnormal reduction or increase in body weight. This is also in agreement with the observation of normal food and water intake of all the animals during the study period.

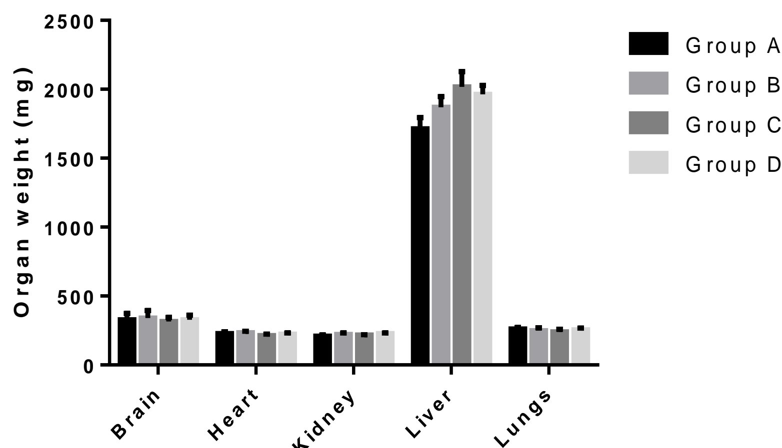

Fig. 2: Effect of Mn-GNP and LDPI formulations on organ weights after 14 days of administration of formulations. Values reported are Mean ± SD, n = 3

The organ weights of the animals were also found to be normal and no significant change in the weights was seen in the experimental animals as compared to the control group animals. The values showed no significant difference ($p < 0.05$) when compared with control group values by using Student's t-test.

The effect on hematological parameters and biochemical parameters of the animals which received the formulations as well as the control group animals are summarized in table 2 and 3 respectively.

Table 2: Effect of Mn-GNP and LDPI formulations on hematological parameters.

Parameter	Group A*	Group B*	Group C*	Group D*
Hb (gm %)	12.13 ± 1.75	12.20 ± 0.96	12.93 ± 1.28	11.40 ± 2.70
RBC (x 10^6/mm^3)	7.36 ± 0.76	7.73 ± 0.24	7.98 ± 0.91	7.65 ± 0.98
WBC (x 10^3/mm^3)	6.60 ± 1.76	6.60 ± 1.11	6.66 ± 1.66	8.26 ± 2.10
PLT (x 10^5/mm^3)	8.11 ± 1.35	7.39 ± 1.32	7.87 ± 1.50	8.31 ± 1.27
PCV (%)	35.33 ± 4.65	34.73 ± 1.35	36.63 ± 3.98	33.80 ± 7.79
MCV (fL)	47.96 ± 1.88	44.93 ± 0.37	46.03 ± 1.60	43.80 ± 5.11
MCH (pg)	16.40 ± 0.86	15.66 ± 1.00	15.80 ± 0.26	14.76 ± 2.02
MCHC (gm / dL)	34.23 ± 0.47	35.06 ± 1.80	35.03 ± 0.73	33.66 ± 1.32
N (%)	31.33 ± 3.05	27.33 ± 9.29	32.00 ± 3.60	26.33 ± 8.38
E (%)	0.66 ± 1.15	0	0.66 ± 0.57	0.33 ± 0.57
L (%)	67.66 ± 3.78	72.00 ± 9.54	67.00 ± 3.60	72.33 ± 8.96
M (%)	0.33 ± 0.58	0.66 ± 0.57	0.33 ± 0.57	1.00 ± 0.00

*Values reported are Mean ± SD, n = 3

Table 3: Effect of Mn-GNP and LDPI formulations on biochemical parameters

Parameter	Group A*	Group B*	Group C*	Group D*
SGOT (IU/L)	199.33 ± 74.00	229.66 ± 19.13	209.00 ± 18.68	207 ± 73.36
SGPT (IU/L)	62.33 ± 3.51	58.66 ± 2.08	54.33 ± 9.07	68.33 ± 31.87
ALP (IU/L)	69.33 ± 34.67	78.33 ± 19.73	85.00 ± 23.64	95.66 ± 18.00
TP (gm / dL)	5.43 ± 0.58	6.13 ± 0.25	5.86 ± 0.70	6.23 ± 0.61
Alb (gm / dL)	3.36 ± 0.47	3.33 ± 0.30	3.73 ± 0.20	3.63 ± 0.20
Glb (gm / dL)	2.06 ± 0.80	2.80 ± 0.55	2.13 ± 0.58	2.60 ± 0.43
BUN (mg / dL)	23.76 ± 3.97	19.66 ± 6.20	19.40 ± 1.67	17.80 ± 3.45
Creat (mg / dL)	0.36 ± 0.05	0.36 ± 0.05	0.33 ± 0.57	0.46 ± 0.20

*Values reported are Mean ± SD, n = 3

All the hematological as well as biochemical parameters of the animals are comparable to those of the corresponding control group animals. The values showed no significant difference ($p < 0.05$) when compared with control group values by using Student's t-test. The liver function as well as kidney function tests gave normal results indicating no hepatotoxicity or nephrotoxicity.

Histopathology

The histopathology of the organs revealed no abnormalities such as necrosis, hemorrhage, lesions, tissue degeneration in any animal. This normal histoarchitecture implies that the formulations are non-toxic. The histopathology images of the animals which received the formulations were comparable to that of the control group animals. The representative histopathology images of the different organs are shown in figures 3 and 4 for nanoparticle and liposome formulations respectively.

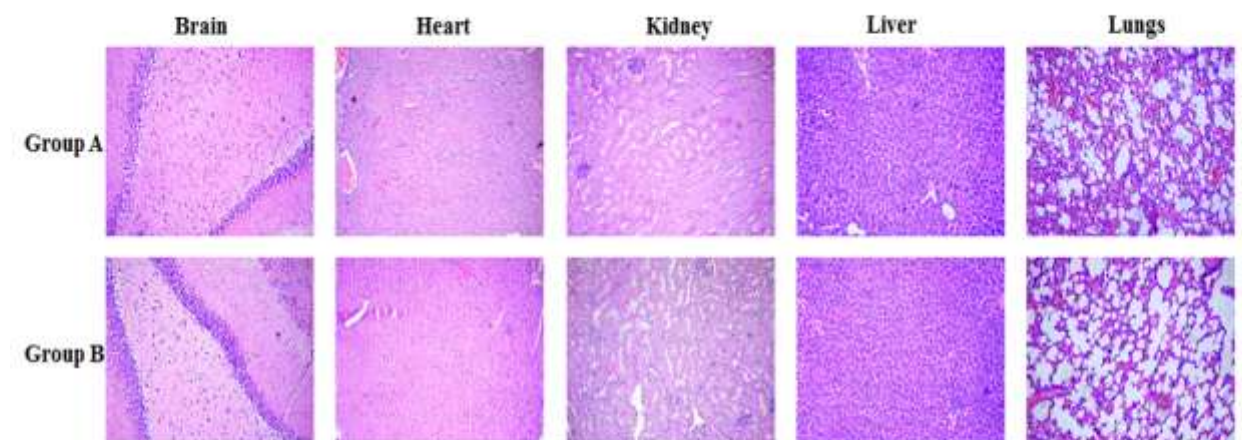

Fig. 3: Comparative histopathology images of brain, heart, kidney, liver and lungs of animals administered intravenous nanoparticles (group B) as compared to control group animals (group A)

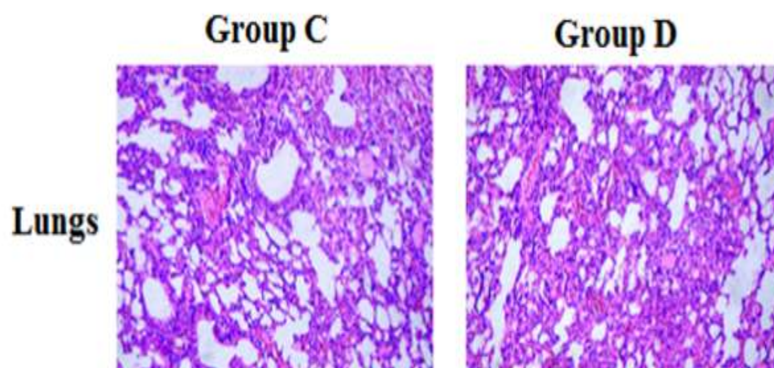

Fig. 4: Comparative histopathology lungs of animals administered dry powder inhalation liposomes (group D) as compared to control group animals (group C)

CONCLUSION

From the findings of this study, we can conclude that the polymeric nanoparticles as well as liposomes of *Glycyrrhiza glabra* extract are safe on acute administration at a dose of 5000 mg/kg. The intended route of administration for polymeric nanoparticles is intravenous whereas that for liposomes is by inhalation. No mortalities or any signs of toxicity were observed throughout the study period implying the safety of the formulations. There was no statistically significant difference in the hematological as well as biochemical parameters of the animals which received the formulations when compared to the control group animals. Histopathology evaluation of the organs showed no changes due to administration of the formulations. This shows that the acute administration of polymeric nanoparticles as well as liposomes of *Glycyrrhiza glabra* extract poses no serious health hazard. Further toxicity profile of the formulations such as sub-acute and chronic toxicity, genotoxocity are required to establish the complete safety assessment of the formulations.

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