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Original Research Article

TOXICOLOGICAL EVALUATION OF KAURENOIC ACID AND ITS NANOLIPOSOMAL FORMULATION IN EXPERIMENTAL ANIMALS

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ABSTRACT

Kaurenoic acid (KA), a diterpenoid with various therapeutic potentials, faces challenges in clinical translation due to poor solubility and bioavailability. Nanoliposomal formulations offer a promising solution to these limitations. This study aimed to evaluate the safety profile of both free kaurenoic acid and its nanoliposomal formulation in experimental animals. Nanoliposomes were prepared using the thin-film hydration method. Acute toxicity test was conducted using Swiss albino. Subacute toxicity was evaluated using male Wistar rats. Animals were orally administered KA and kaurenoic acid loaded nanoliposomes (KALNL) at doses of 4, 40, and 400 mg/kg daily for seven consecutive days. Haematological, biochemical analyses as well as histopathological examinations of the liver and kidneys were also performed. Acute toxicity study showed no lethal outcomes or behavioral changes up to 5000 mg/kg for either KA or KALNL, indicating an oral LD_{50} greater than 5000 mg/kg. Body weight gain remained normal and comparable to controls. The subacute toxicity assessment revealed no mortality, gross pathological findings, or overt toxicity in any of the treated groups in haematological and biochemical parameters. Histopathological examination confirmed preserved hepatic and renal architecture, with no signs of necrosis, fatty degeneration, inflammatory cell infiltration, degeneration, or congestion in the liver and kidney sections, even at higher doses. Both KA and KALNL demonstrated a favorable safety profile during acute and subacute toxicity evaluations in experimental animals, suggesting their potential for further development in therapeutic applications.

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INTRODUCTION

Kaurenoic acid is a diterpenoid that has been isolated from numerous plant species and is recognized for its potential therapeutic applications [1]. It has been demonstrated to possess antiallergic, immunosuppressive, anticancer, anti-inflammatory, antimicrobial activities amongst many others [2]. Beyond these specific actions, kaurenoic acid is also being investigated for its anticancer, antidiabetic, hepatoprotective and neuroprotective properties, further expanding its therapeutic potential [3-7]. However, the poor solubility and bioavailability of kaurenoic acid have hindered its clinical translation.

Nanotechnology has emerged by providing innovative drug delivery systems that can overcome the limitations of poor

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solubility and low bioavailability. Lipid nanocarriers have emerged as a promising drug delivery system due to their ability to enhance drug solubility, protect therapeutic agents from degradation, improve bioavailability, and enable controlled release [8]. Specifically, nanoliposomes, a type of lipid nanoparticle, have demonstrated significant potential in overcoming challenges associated with poorly water-soluble therapeutic agents, including natural products, by facilitating improved solubility, tissue distribution, and prolonged retention within biological systems [9,10]. This enhanced efficacy is attributed to their biocompatible and biodegradable nature, coupled with their nanoscale dimensions, allowing for diverse applications in cancer nanotherapy, gene delivery, and nutraceuticals [11].

The safety and biodistribution of these nanocarriers are critical for their clinical translation, necessitating comprehensive in vivo toxicity evaluations, including repeated-dose studies, to assess potential adverse effects and tissue accumulation [12]. While acute and subacute toxicity studies often employ low dosages, chronic administration of nanoparticles, especially in the context of chronic diseases, requires extensive investigation into long-term safety profiles across various physiological parameters [12,13]. Given the escalating use of nanotechnology in biomedical applications, particularly for drug delivery systems, a thorough understanding of the toxicity of nanocarriers, including their physicochemical properties, administration routes, and dosages, becomes paramount to ensure their safe and effective deployment [14]. Furthermore, the long-term biological interactions of inorganic nanoparticles, encompassing their accumulation, degradation, and potential for delayed toxicities, remain largely unexplored, emphasizing the need for comprehensive subchronic and chronic toxicity assessments beyond acute observations [14]. This gap in knowledge underscores the importance of evaluating nanoliposomal formulations of bioactive compounds like kaurenoic acid, particularly concerning their long-term effects and systemic safety following repeated administration in animal models [15].

Scientific data are lacking to confirm the safety profile of repeated exposure of KA. In addition, there is no report on safety study of nanoliposomal formulation of kaurenoic acid. Thus, the present study was designed to evaluate the safety profile of kaurenoic acid and kaurenoic acid loaded nanoliposomes.

MATERIALS AND METHODS

Chemicals and reagents

Soy phosphatidylcholine was procured from Avanti polar Lipids (Alabama, USA), cholesterol and kaurenoic acid were obtained from Cayman Chemicals (Michigan, USA), ethanol and Tween 80 were obtained from Loba Chemicals (Mumbai, India).

Nanoliposome preparation and drug loading

Nanoliposomes were produced by the thin-film hydration method [16,17]. Briefly, soy phosphatidylcholine and cholesterol (7.5:2.5) were dissolved in 10 ml absolute ethanol in a round-bottom flask, and the organic solvent was removed using a rotary evaporator until a thin film was formed on the walls. The dried lipid film was then hydrated at 70 °C using 250 mM ammonium sulfate solution (pH 5.4). The hydrated suspension was subjected to stepwise extrusion through a series of polycarbonate membrane filters with progressively smaller pore sizes (0.8, 0.4, 0.2 and 0.1 µm) using a mini-extruder (Avanti polar lipids, USA). Each extrusion step was repeated 5–10 times at 70 °C. KA was incorporated into preformed nanoliposomes using a direct post-insertion method [16]. Briefly, a 500 mg kaurenoic acid was accurately weighed and dissolved in 1 ml absolute ethanol, followed by gentle warming to 50–60 °C. The preformed nanoliposomes were also prewarmed to the same temperature. The ethanolic solution of KA was added dropwise to the nanoliposome dispersion under continuous stirring at 70 °C. Physical properties of the prepared liposome formulation including size, polydispersity index (PDI), and surface charge (zeta potential) were measured by the dynamic light scattering (DLS) method using a Zetasizer (Nano-ZS, Malvern, UK) at room temperature.

Experimental animals

Nine Adult Swiss albino mice (25 – 30 g) of both sexes and Forty-five male Wistar rats weighing 130-150 g were obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. The animals were kept under standard laboratory conditions at a temperature of (25 ± 2) ºC, with a 12:12 h light:dark cycle and 45%–55% relative humidity. The animals were fed with standard rat pellet diet and water ad libitum. The study was carried out and the animals were handled according to the guidelines of the National Code of Conduct for Animal Research Ethics (NCARE). All experimental procedures were approved by the Faculty of Pharmaceutical Sciences Research Ethics Committee with approval number FPSRA/UNN/23/00031. All the experiments were performed according to the institutional guidelines.

Acute toxicity test

Lorke's method [18] of acute toxicity test determination was adopted in this study. Briefly, adult Swiss albino mice (25-30 g) of both sexes were allotted into three groups of three animals per group in the first phase. Groups A-C received orally 10, 100 and 1000 mg/kg of KA respectively. The animals were constantly observed for the first 2 h, intermittently for the next 4 h and then overnight for signs of

toxicity. The number of mortality per group was recorded at the end of 24 h. From the results obtained, the second phase of the acute toxicity test was performed using doses of 1600, 2900 and 5000 mg/kg KA administered to three groups of one animal per group while the fourth animal received the vehicle (5% Tween 80, 2 ml/kg). The animals were again observed for the first 2 h, intermittently for the next 4 h and then overnight for signs of toxicity as well as mortality. Similar protocol was adopted for KALNL. At the end of the study, the LD50 was calculated as the geometric mean of the highest non-lethal dose and the lowest lethal dose using the equation:

$$LD50 = \sqrt{D0} \times \sqrt{D1}$$ ------- Eqn 1

Where D0 is the highest non-lethal dose

D_1 is the lowest lethal dose

Subacute toxicity study

The subacute oral toxicity was conducted in accordance to the Organization for Economic Cooperation and Development (OECD) Test Guidelines 407 with modifications [19]. Adult male Wistar rats (40) were randomly assigned into eight groups (n = 5 rats/group) as follows: Group 1 served as normal control, Group 2 received the vehicle (5% Tween 80, 2 ml/kg); Group 3 received blank nanoliposomes (2 ml/kg); Groups 4–6 received free KA at 4, 40, and 400 mg/kg respectively; Groups 7–9 received KALNL at 4, 40, and 400 mg/kg respectively. Animals were treated orally once daily consecutively for 7 days. The animals were observed daily for clinical signs and mortality. At the end of the experiment, animals were fasted overnight and anaesthetized using ketamine 80 mg/kg and xylazine 10 mg/kg, intraperitoneally and blood was collected by cardiac puncture for haematological and biochemical analyses. After euthanasia, target organs were collected for histopathological examination.

Relative organ weight determination

On the 9th day, animals were euthanized following an overnight fast. Major organs including the liver, kidneys, heart, and lungs were carefully excised, cleared of adherent tissues, blotted dry, and weighed to the nearest gram. The relative organ weight of each organ was calculated using the formula:

$$\text{Relative organ weight}(\%) = \frac{\text{Absolute organ weight}\,(g)}{\text{Body weight of rat on sacrifice day}\,(g)} \times 100$$ ------ Eqn 2

Blood sample collection

Blood samples were collected from rats via cardiac puncture under appropriate anesthesia. The samples were divided into two portions: one collected in plain tubes (non-heparinized) for serum preparation and the other in heparinized tubes for plasma collection. All samples were centrifuged at 3000 × g for 10 mins using a bench-top centrifuge. The resulting serum and plasma were carefully separated and transferred into fresh, labeled plain tubes. The processed samples were subsequently used for haematological and biochemical analyses.

Haematological analyses

Packed cell volume (PCV), white blood count (WBC), neutrophils, lymphocytes, monocytes, eosinophils, haemoglobin (Hb), platelets, red blood cell (RBC) were determined using semi-automated chemistry analyzer supplied by Erba diagnostics, Germany.

Biochemical analyses

Biochemical analyses carried out include measurement of the activities of liver function markers such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), as well as total protein, albumin, globulin and bilirubin, renal function marker s such as urea and creatinine, serum electrolytes such as sodium, potassium, calcium, phosphorus and chloride, lipid profiles such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), cholesterol, and triacylglycerol. In addition, total acid phosphatase, non-prostatic acid phosphatase and prostatic acid phosphatase levels were measured.

Histopathological examination

The histology alterations in the tissues of the vital organs including the kidneys and liver were evaluated using hematoxylin & eosin (H&E) staining. Briefly, a small piece of fresh tissue sample was cut and immersion fixed in 10% buffered formalin. Then, the formalin-preserved specimens were gradually dehydrated, embedded in paraffin, cut into layers with 5-μm thickness, deparaffinized by p-xylene, rehydrated by ethanol, rinsed by water, and eventually stained by the H&E approach. The histopathological changes of H&E stained samples were evaluated by an expert pathologist, using light microscopy (Axiocam microscope) supplied with a digital camera under a magnification of 400×.

Statistical Analysis

Data obtained were expressed as mean ± standard error of mean (SEM) and were analyzed using Graphpad prism version 9.5 software. The data were subjected to One-way ANOVA followed by Tukey's multiple comparison. The data were considered significant at $p < 0.05$.

RESULTS

Nanoliposomal encapsulation of kaurenoic acid

Nanoliposomes were prepared by the thin-film hydration method. KALNL had a mean diameter of 43.96 ± 3.10 nm, polydispersity index (PDI) of 0.42 ± 0.79, and zeta potential -36.42 ± 3.50 mV. The entrapment efficiency was 89.12%.

Acute toxicity studies

At the tested doses of up to 5000 mg/kg, neither KA nor KALNL produced lethal outcomes or induced any behavioral

changes or signs of toxicities during the observation period. Consequently, the calculated oral LD_{50} value for KA and KALNL was determined to be greater than 5000 mg/kg.

Subacute toxicity studies

The repeated-dose oral toxicity assessment demonstrated a lack of mortality and an absence of gross pathological findings across all treated groups. All test and control animals maintained a healthy status for the duration of the study.

Effect of KA and KALNL on relative organ weight

Relative organ weights of the heart, liver, kidney, and lungs remained consistent across all treatment groups, showing no significant deviations from the control. Both KA and KALNL at all tested doses did not induce abnormal changes throughout the 7-day treatment period (Table 1)

Effect of KA and KALNL on body weight

Body weight gain across all groups was normal and comparable to controls, demonstrating no overt toxicity. The absence of significant or dose-dependent effects on growth indicates both KA and KALNL were well-tolerated and did not adversely impact nutritional status (Table 2).

Table 1: Effect of KA and KALNL on relative organ weight (%)

Treatment	Heart	Liver	Kidney	Lungs
Normal control	0.64 ± 0.08	8.04 ± 0.18	0.78 ± 0.14	1.45 ± 0.26
Blank nanoliposomes	0.62 ± 0.02	8.10 ± 0.07	0.64 ± 0.11	1.13 ± 0.21
5% Tween 80 (2 ml/kg)	0.66 ± 0.06	7.98 ± 0.09	0.68 ± 0.18	1.28 ± 0.26
KALNL (4 mg/kg)	0.63 ± 0.09	8.12 ± 0.05	0.60 ± 0.24	1.35 ± 0.29
KALNL (40 mg/kg)	0.62 ± 0.09	8.10 ± 0.10	0.70 ± 0.14	1.39 ± 0.29
KALNL (400 mg/kg)	0.63 ± 0.03	8.08 ± 0.12	0.68 ± 0.19	1.38 ± 0.23
KA (4 mg/kg)	0.62 ± 0.02	8.00 ± 0.11	0.72 ± 0.10	1.30 ± 0.22
KA (40 mg/kg)	0.64 ± 0.08	8.06 ± 0.19	0.70 ± 0.18	1.29 ± 0.28
KA (400 mg/kg)	0.64 ± 0.02	8.02 ± 0.06	0.69 ± 0.21	1.32 ± 0.30

Values are expressed as mean ± SEM; (n = 5); KA = kaurenoic acid, KALNL = kaurenoic acid loaded nanoliposome

Table 2: Effect of KA and KALNL on body weight (g)

Treatment	Day 0	Day 7
Normal control	149.4 ± 14.98	165.9 ± 19.38
Blank nanoliposomes	142.0 ± 15.22	155.0 ± 16.67
5% Tween 80 (2 ml/kg)	147.2 ± 13.96	163.1 ± 17.66
KALNL (4 mg/kg)	135.6 ± 10.49	152.9 ± 12.58
KALNL (40 mg/kg)	134.6 ± 5.69	149.3 ± 6.98
KALNL (400 mg/kg)	137.3 ± 9.43	157.4 ± 15.23
KA (4 mg/kg)	135.2 ± 8.22	156.9 ± 7.21
KA (40 mg/kg)	138.3 ± 4.68	159.8 ± 8.32
KA (400 mg/kg)	136.0 ± 3.42	153.4 ± 6.52

Values are expressed as mean ± SEM; (n = 5); KA = kaurenoic acid, KALNL = kaurenoic acid loaded nanoliposome

Effect of KA and KALNL on haematological parameters

The haematological analyses indicated a general safety profile for both free KA and KALNL, as all major parameters (RBC, haemoglobin, PCV, WBC, platelets) remained within normal physiological limits. Although a mild, dose-dependent shift in the leukocyte differential was observed—characterized by a slight decrease in lymphocytes and an increase in heterophils and monocytes at the highest KALNL dose (Table 3).

Effect of KA and KALNL on biochemical parameters

Biochemical analyses revealed a hepatoprotective potential for KA, evidenced by significantly ($p < 0.01$, 0.001) reduced AST levels at 40 and 400 mg/kg, with no concomitant changes in ALT or ALP to indicate hepatic injury. There were significant reductions in urea ($p < 0.05$; KALNL 400 mg/kg) and phosphorus ($p < 0.01$; KA 400 mg/kg) compared to normal control. All other parameters, including renal function markers, electrolytes, and lipids, remained within normal physiological limits. Total acid phosphatase (TAP) remained within normal limits, though KALNL at higher doses slightly elevated values compared to normal control. Non-prostatic acid phosphatase (Non-PAP) also increased with KALNL at 40–400 mg/kg whereas prostatic acid phosphatase (PAP) generally decreased across KA and KALNL groups relative normal control (Table 4).

Table 3: Effects of KA and KALNL on haematological parameters

Parameters	Normal control	Blank nanoliposomes	5% Tween 80 (2 ml/kg)	KALNL (4 mg/kg)	KALNL (40 mg/kg)	KALNL (400 mg/kg)	KA (4 mg/kg)	KA (40 mg/kg)	KA (400 mg/kg)
RBC (10^6/µl)	7.17 ± 0.63	6.56 ± 0.13	7.86 ± 0.49	7.58 ± 0.54	6.51 ± 0.24	7.09 ± 0.44	7.65 ± 0.61	7.64 ± 0.65	7.88 ± 0.34
Haemoglobin (g/dl)	15.89 ± 0.42	15.20 ± 0.21	15.82 ± 0.75	15.00 ± 0.49	15.38 ± 0.82	14.83 ± 0.65	15.44 ± 0.70	14.70 ± 0.21	15.24 ± 0.83
PCV (%)	37.37 ± 2.58	35.63 ± 0.74	41.47 ± 1.92	40.43 ± 2.29	34.63 ± 1.57	38.23 ± 1.39	40.33 ± 3.06	41.53 ± 3.68	41.87 ± 2.31
Total WBC (10^3/µl)	11.90 ± 0.15	9.10 ± 0.55	10.73 ± 1.78	10.73 ± 2.04	12.73 ± 2.31	9.80 ± 1.03	10.17 ± 1.86	10.93 ± 0.69	12.30 ± 0.93
Platelets (10^3/µl)	185.0 ± 18.03	171.8 ± 5.85	181.7 ± 9.28	195.0 ± 7.64	173.3 ± 4.41	185.0 ± 16.07	190.0 ± 11.55	183.3 ± 10.14	183.3 ± 7.27
Lymphocytes (%)	82.33 ± 2.33	77.00 ± 2.00	73.33 ± 1.86	69.33 ± 5.61	73.00 ± 4.58	77.33 ± 0.88	80.33 ± 1.67	80.67 ± 2.40	80.33 ± 4.26
Heterophils (%)	14.33 ± 2.60	20.67 ± 2.19	23.00 ± 1.53	28.00 ± 5.29	21.67 ± 4.63	18.00 ± 2.08	17.00 ± 1.53	17.00 ± 2.08	16.00 ± 3.79
Eosinophils (%)	1.67 ± 0.33	1.00 ± 0.00	2.00 ± 0.58	1.33 ± 0.33	2.00 ± 1.00	1.68 ± 0.33	1.00 ± 0.00	1.33 ± 0.33	2.00 ± 0.58
Monocytes (%)	1.68 ± 0.33	1.33 ± 0.33	1.68 ± 0.33	1.33 ± 0.33	3.33 ± 0.88	3.00 ± 1.16	1.67 ± 0.33	1.00 ± 0.00	1.68 ± 0.33
Abs lymphocytes (10^3/µl)	9.80 ± 0.34	7.01 ± 0.46	7.81 ± 1.17	7.38 ± 1.33	9.28 ± 1.64	7.56 ± 0.71	8.17 ± 1.56	8.97 ± 0.37	9.81 ± 0.37
Abs eosinophils (10^3/µl)	0.20 ± 0.04	0.09 ± 0.01	0.23 ± 0.08	0.14 ± 0.03	0.30 ± 0.19	0.17 ± 0.04	0.10 ± 0.02	0.15 ± 0.05	0.25 ± 0.08
Abs monocytes (10^3/µl)	0.20 ± 0.04	0.12 ± 0.02	0.18 ± 0.05	0.16 ± 0.07	0.47 ± 0.20	0.29 ± 0.10	0.18 ± 0.06	0.11 ± 0.01	0.21 ± 0.04

Values are expressed as mean ± SEM; (n = 5); KA = kaurenoic acid, KALNL = kaurenoic acid loaded nanoliposome, RBC = red blood cells, PCV = packed cell volume, WBC = white blood cell, Abs = absolute

Table 4: Effects of KA and KALNL on biochemical parameters

Parameters	Normal control	Blank nanoliposomes	5% Tween 80 (2 ml/kg)	KALNL (4 mg/kg)	KALNL (40 mg/kg)	KALNL (400 mg/kg)	KA (4 g/kg)	KA (40 mg/kg)	KA (400 mg/kg)
AST (IU/L)	111.20 ± 15.44	92.99 ± 5.13	85.24 ± 4.19	86.02 ± 4.31	81.75 ± 5.37	85.46 ± 10.65	81.36 ± 3.03	75.55 ± 0.39*	65.26 ± 1.11**
ALT (IU/L)	25.17 ± 3.02	19.82 ± 2.01	26.38 ± 5.58	23.92 ± 1.14	21.17 ± 0.91	17.59 ± 0.91	23.89 ± 0.82	25.51 ± 3.37	23.12 ± 1.29
ALP (IU/L)	52.46 ± 4.95	54.55 ± 3.84	51.89 ± 0.64	51.17 ± 1.25	53.38 ± 1.84	50.87 ± 0.52	53.48 ± 5.27	42.57 ± 1.41	49.38 ± 0.97
Total Bilirubin (mg/dl)	0.55 ± 0.15	0.44 ± 0.12	0.42 ± 0.04	0.44 ± 0.02	0.38 ± 0.05	0.31 ± 0.01	0.38 ± 0.05	0.40 ± 0.09	0.27 ± 0.02
Total proteins (mg/dl)	70.25 ± 1.79	66.02 ± 4.24	69.93 ± 2.56	67.27 ± 1.89	72.10 ± 0.68	69.22 ± 0.85	66.02 ± 1.41	66.56 ± 0.43	70.68 ± 0.66
Albumin (mg/dl)	37.83 ± 0.68	36.37 ± 0.83	34.85 ± 2.39	35.28 ± 0.63	37.01 ± 0.55	37.84 ± 0.62	38.28 ± 1.27	33.93 ± 1.74	38.43 ± 0.72
Globulin (mg/dl)	32.42 ± 1.29	29.65 ± 4.42	35.08 ± 1.97	31.99 ± 1.43	35.09 ± 1.22	31.37 ± 1.38	27.74 ± 2.67	32.63 ± 1.58	32.26 ± 0.19
Creatinine (mg/dl)	0.53 ± 0.02	0.58 ± 0.16	0.57 ± 0.03	0.56 ± 0.02	0.57 ± 0.11	0.50 ± 0.03	0.63 ± 0.04	0.53 ± 0.12	0.50 ± 0.02
Urea (mg/dl)	34.67 ± 1.38	32.57 ± 0.68	32.99 ± 0.16	33.51 ± 0.59	32.65 ± 0.22	29.26 ± 1.91*	31.27 ± 1.03	30.81 ± 0.93	30.43 ± 0.64
Sodium (mEq/L)	137.9 ± 4.60	140.1 ± 1.92	123.4 ± 5.80	131.5 ± 6.85	125.2 ± 4.51	136.2 ± 3.99	135.2 ± 2.48	134.3 ± 5.18	136.9 ± 2.86
Potassium (mEq/L)	6.24 ± 0.62	5.71 ± 0.56	6.23 ± 1.02	5.60 ± 0.63	6.26 ± 1.14	5.79 ± 0.59	5.73 ± 0.36	5.87 ± 0.78	5.13 ± 0.60
Chloride (mEq/L)	85.48 ± 0.79	88.52 ± 1.41	83.78 ± 1.09	88.42 ± 2.84	86.48 ± 1.53	82.93 ± 3.16	85.98 ± 1.34	86.98 ± 2.46	82.54 ± 6.19
Calcium (mg/dl)	9.83 ± 0.56	10.12 ± 0.43	10.29 ± 0.23	10.25 ± 0.23	10.48 ± 0.38	9.87 ± 0.13	10.48 ± 0.12	10.00 ± 0.59	10.70 ± 0.36

Phosphorus (mg/dl)	16.13 ± 0.93	16.47 ± 0.07	18.33 ± 0.93	16.27 ± 1.16	14.60 ± 0.20	14.20 ± 0.90	15.67 ± 0.55	15.00 ± 0.61	13.53 ± 0.35**
Total cholesterol (mg/dl)	101.6 ± 11.76	79.72 ± 1.61	88.31 ± 6.91	90.56 ± 1.63	91.18 ± 2.16	78.18 ± 3.93	110.3 ± 1.95	101.8 ± 11.14	101.9 ± 2.22
Triglycerides (mg/dl)	160.8 ± 21.63	175.3 ± 12.80	175.8 ± 7.75	181.3 ± 9.75	176.3 ± 11.74	150.3 ± 11.81	176.3 ± 6.61	162.3 ± 12.95	148.8 ± 6.61
HDL (mg/dl)	53.30 ± 7.74	46.70 ± 4.52	42.84 ± 3.41	52.82 ± 6.22	37.20 ± 6.43	43.96 ± 5.38	48.95 ± 3.54	51.05 ± 8.39	38.49 ± 3.47
VLDL (mg/dl)	32.16 ± 4.33	35.05 ± 2.56	35.15 ± 1.55	36.25 ± 1.95	35.25 ± 2.35	30.06 ± 2.36	35.26 ± 1.32	32.46 ± 2.59	29.76 ± 1.32
LDL (mg/dl)	48.31 ± 4.10	33.02 ± 6.10	45.47 ± 4.16	37.74 ± 4.89	53.98 ± 7.22	34.22 ± 1.97	61.35 ± 5.46	50.77 ± 5.80	63.43 ± 5.42
TAP (IU/L)	8.82 ± 1.15	6.94 ± 0.35	7.62 ± 0.82	8.19 ± 0.10	10.80 ± 1.10	10.58 ± 1.68	6.48 ± 0.35	5.00 ± 0.64	6.88 ± 0.21
Non-PAP (IU/L)	3.96 ± 0.78	4.64 ± 0.53	5.04 ± 0.80	5.04 ± 0.25	6.54 ± 0.36	7.22 ± 0.60	4.24 ± 0.95	3.44 ± 0.70	4.47 ± 0.36
PAP (IU/L)	4.86 ± 0.84	2.30 ± 0.21	2.58 ± 0.95	3.15 ± 0.15	4.27 ± 1.13	3.36 ± 1.29	2.24 ± 0.97	1.56 ± 0.47	2.41 ± 0.41

Values are expressed as mean ± SEM; (n = 5); * = $p < 0.05$, ** = $p < 0.01$ for treated groups vs. normal control. KA = kaurenoic acid, KALNL = kaurenoic acid loaded nanoliposome, AST = aspartate transaminase, ALT = alanine transaminase, ALP = alkaline phosphatase, HDL = high density lipoprotein, VLDL = very low density lipoprotein, LDL = low density lipoprotein, TAP = total acid phosphatase, Non-PAP = non-prostatic acid phosphatase, PAP = prostatic acid phosphatase

Histopathological examination

The liver sections from the normal control and treated groups showed preserved hepatic architecture with intact hepatocytes and central veins. In groups treated with both free kaurenoic acid (KA) and kaurenoic acid-loaded nanoliposomes (KALNL), no significant necrosis, fatty degeneration, or inflammatory cell infiltration was observed, even at higher doses (Figure 1). Similarly, the kidney photomicrographs revealed normal glomerular and tubular structures with no evidence of degeneration, congestion, or interstitial inflammation (Figure 2).

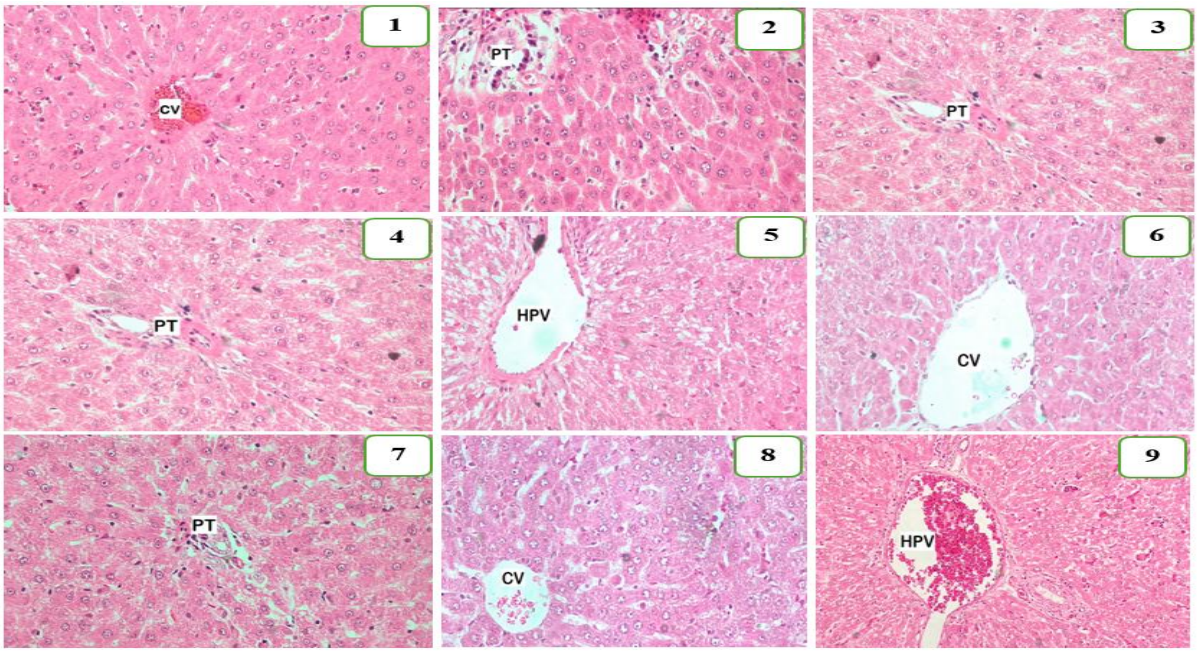

Figure 1: Photomicrograph of the liver showing the central vein (CV), portal triad (PT) or hepatic portal vein (HPV) (as applied) and normal hepatocytes. 1= Normal control; 2= Blank nanoliposomes (2 ml/kg); 3= 5% Tween 80 (2 ml/kg); 4= KALNL (4 mg/kg); 5= KALNL (40 mg/kg); 6= KALNL (400 mg/kg); 7= KA (4 mg/kg); 8= KA (40 mg/kg); 9= KA (400 mg/kg); magnification= ×400

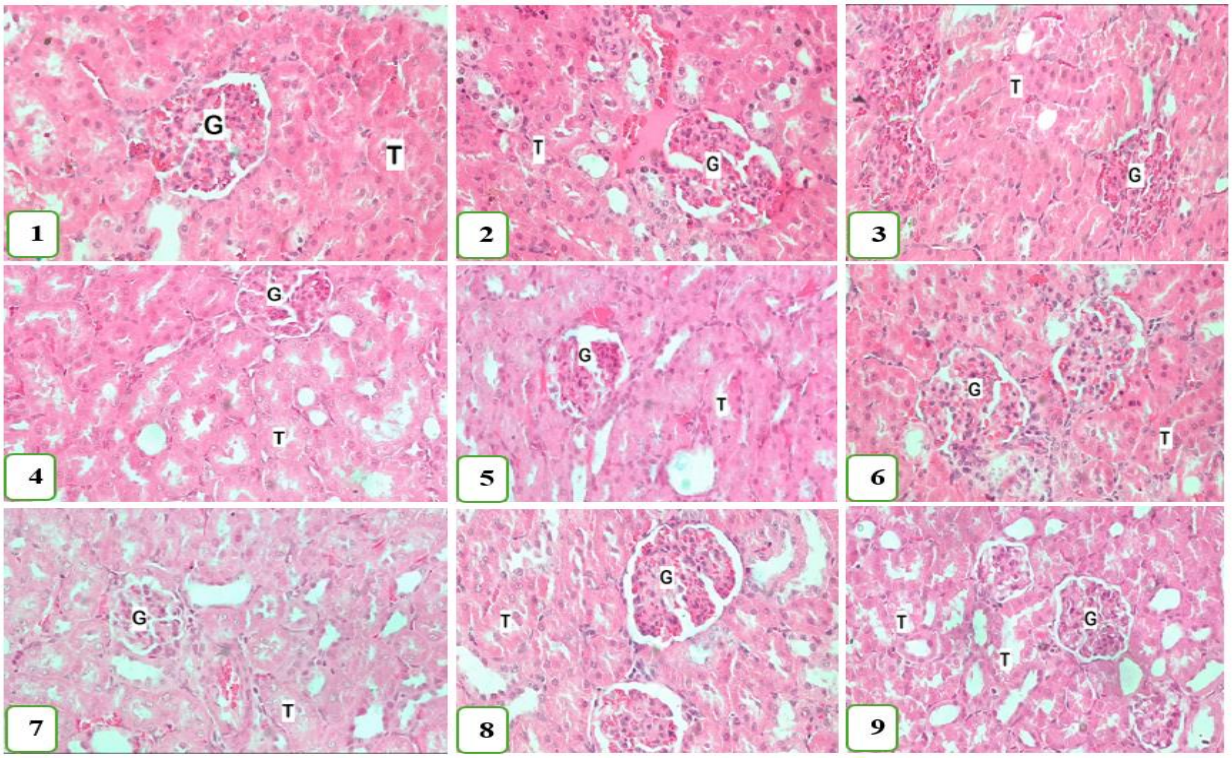

Figure 2: Photomicrograph of the kidney showing normal glomerulus (G) and renal tubules (T). 1= Normal control; 2= Blank nanoliposomes (2 ml/kg); 3= 5% Tween 80 (2 ml/kg); 4= KALNL (4 mg/kg); 5= KALNL (40 mg/kg); 6= KALNL (400 mg/kg); 7= KA (4 mg/kg); 8= KA (40 mg/kg); 9= KA (400 mg/kg); magnification= ×400

DISCUSSION

The present study aimed to address a notable gap in the scientific literature concerning the safety profile of KA and, more specifically, its nanoliposomal formulation following repeated exposure in experimental animal models. A comprehensive understanding of the toxicity of such formulations is critical for their safe and effective deployment in biomedical applications.

Our findings demonstrated a favorable safety profile for both free KA and its nanoliposomal formulation. In acute toxicity assessments, neither KA nor KALNL induced lethal outcomes or behavioral changes in mice at a high oral dose of 5000 mg/kg, indicating an oral LD_{50} greater than this value. This suggests a low acute toxicity potential for both forms of kaurenoic acid which is in agreement to the toxicity classification reported by Loomis and Hayes [20].

Furthermore, the subacute toxicity study, which involved repeated oral administration, revealed a complete absence of mortality and gross pathological findings across all treated groups. Animals maintained a healthy status throughout the study, exhibiting normal body weight gain comparable to control groups, which serves as a crucial indicator of overall well-being and lack of overt systemic toxicity. Generally, a reduction in body weight and internal organ weights is a simple and sensitive index of toxicity after exposure to potentially toxic substances [21,22]. In the present study, KA and KALNL did not affect body nor organ weight as compared to the normal control group, which suggests that KA and KALNL did not disturb rat growth.

The evaluation of haematological parameters is of great importance in determining the health status of an individual [23]. From our findings, KA and KALNL did not cause significant deviations in RBC, haemoglobin, PCV, WBC, or platelet counts compared to controls, suggesting preservation of normal hematopoiesis and immune competence. Minor variations in lymphocyte and heterophil percentages remained within physiological ranges, indicating absence of immunotoxicity. These findings highlight the haematological safety of both free KA and KALNL, supporting their suitability for further therapeutic evaluation.

Elevated levels of serum transaminase enzymes (ALT, AST and ALP) are clear indications of hepatic impairment in animals [24]. In this study, KA and KALNL did not produce significant elevations in ALT, ALP, total bilirubin, total protein, or albumin compared to controls, indicating no hepatocellular injury or impaired synthetic function. Notably, high-dose KA reduced AST activity, which may reflect a protective or enzyme-stabilizing effect rather than toxicity. These results suggest that both free KA and KALNL are safe on the hepatocytes and may even confer liver-protective effects. In addition, KA and KALNL produced no significant alterations in total cholesterol, triglycerides, HDL, VLDL, or LDL compared to controls, indicating no dyslipidemic effect. These results suggest both formulations maintain lipid homeostasis and are unlikely to pose cardiovascular risks.

Renal function tests are useful for identifying the presence of renal disease, monitoring the response of kidneys to treatment, and determining the progression of renal disease [25]. The most commonly used endogenous marker for assessing glomerular function is creatinine [26]. Urea, or blood urea nitrogen (BUN), is a nitrogen-containing compound formed in the liver as the end product of protein metabolism and the urea cycle [27]. The accumulation of creatinine, urea, uric acid, and electrolytes in the body reflect impaired renal clearance and are key indicators of kidney damage [28]. Disturbances in electrolyte balance, particularly sodium, potassium, chloride, and magnesium, further signify renal injury and compromised homeostatic function [29]. From our findings, KA and KALNL produced no significant alterations in creatinine, urea, sodium, potassium, chloride, or calcium compared to controls, indicating preserved renal function and electrolyte balance. The mild reduction in urea observed with high-dose KALNL remained within physiological range, suggesting absence of nephrotoxicity. These findings signify that both free KA and KALNL were well tolerated by the kidney, supporting their safety in preclinical use.

Elevated serum PAP activity reflects prostate gland damage, inflammation, or carcinoma, making it useful in toxicological and pharmacological studies in animal models [30]. PAP generally decreased across KA and KALNL groups, indicating no adverse stimulation of prostate-specific activity. Total acid phosphatase (TAP) remained within normal limits, though KALNL at higher doses slightly elevated values compared to control. Non-prostatic acid phosphatase (Non-PAP) also increased with KALNL at 40–400 mg/kg, suggesting mild systemic enzyme induction.

The histopathological examination provided further robust evidence of the safety of KA and KALNL. Analysis of liver sections showed preserved hepatic architecture with intact hepatocytes and central veins in all treated groups, even at the highest doses. No necrosis, fatty degeneration, or inflammatory cell infiltration was observed in the liver. Similarly, kidney photomicrographs confirmed normal glomerular and tubular structures, with no signs of degeneration, congestion, or interstitial inflammation. These results are particularly significant as the liver and kidneys are primary organs involved in drug metabolism and excretion, making them highly susceptible to xenobiotic-induced toxicity. The integrity of these organs suggested that both free KA and KALNL did not induce significant organ damage under the tested conditions.

The physicochemical properties of the nanoliposomal formulation, including a mean diameter of 43.96 ± 3.10 nm, a polydispersity index of 0.42 ± 0.79, a zeta potential of -36.42 ± 3.50 mV, and an entrapment efficiency of 89.12%, indicate a well-characterized and stable delivery system. The observed safety of KALNL is crucial, as nanocarriers themselves can sometimes pose toxicity concerns, underscoring the importance of evaluating their long-term effects and systemic safety. This study contributed valuable data to the limited existing knowledge regarding the safety of nanoliposomal formulations of bioactive compounds, particularly in the context of repeated administration.

CONCLUSION

The findings of this study demonstrated that both KA and its nanoliposomal formulation were well-tolerated in experimental animals, exhibiting a high safety margin in both acute and subacute toxicity evaluations. These findings are encouraging for the potential clinical translation of KA, particularly when encapsulated into nanoliposomes to improve its pharmacological properties. Future research could explore chronic toxicity, pharmacokinetic profiles, and efficacy studies to further substantiate the safety and therapeutic utility of KA and KALNL.

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AUTHORS' CONTRIBUTION

IEP, PAA, HNA, BCO, MOA, CSN, PME, SA, TCA, AAA, PAA: Conceptualized the problem and designed the study.
IEP, TCA, BCO, PAA, AAA: Drafted the manuscript. IEP, CSN, TCA: Performed statistical analysis on the data generated. IEP, PAA, MOA, CSN, TCA, Proofread the manuscript before final submission

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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