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Hepatoprotective and anti-carcinogenic potential of mercury-containing drug, *Lokanatha* Rasa against hepatocellular carcinoma: In vitro cytotoxic, anti-inflammatory and genotoxic evaluation

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent and lethal malignancies worldwide and it is one of the top causes of cancer-related deaths. It is also closely linked to chronic inflammation, oxidative damage and persistent hepatocellular injury, which are all factors that contribute to the development of cancer. The conventional treatments for cancer, such as chemotherapy, radiation therapy and targeted biologic therapies, are often plagued by systemic toxicity, resistance and poor response rates, thus underlining the need for multi-targeted therapies. Rasa Shastra is a specialized branch of Ayurveda that lists classical herbo-mineral and mercurial compounds for the treatment of liver ailments (*Yakrit Vikara*) and chronic metabolic disorders. *Lokanatha Rasa* (LKN) is a classical mercurial drug that is made up of purified mercury (*Shoditha Parada*), sulphur (*Shoditha Gandhaka*), calcined minerals, and cowry shells, which are further processed by classical methods of purification (*Shodhana*) and incineration (*Marana*). This work assessed the in vitro cytotoxic, anti-inflammatory and genotoxic properties of LKN on HepG2 human hepatocellular carcinoma cells. Cytotoxicity was assessed by the MTT assay, anti-inflammatory activity by the Human Red Blood Cell (HRBC) membrane stabilization assay and genotoxicity by the comet assay. LKN exhibited dose-dependent cytotoxicity with an IC_{50} of 1.6 mg/mL, while anti-inflammatory activity increased with concentration, although diclofenac had higher membrane-stabilizing activity. The comet assay showed DNA strand breaks at medium and high concentrations, indicating possible apoptosis induction. The combined results suggest that LKN has multi-targeted biological activity, which includes moderate cytotoxicity, membrane-stabilizing anti-inflammatory activity, and DNA damage. These findings offer preliminary scientific evidence for the traditional use of Rasa Shastra drugs in liver diseases and emphasize the importance of traditional processing methods in improving their efficacy. LKN has potential as an adjuvant in HCC treatment and requires further investigations

Keywords: Anti-inflammatory activity, comet assay, cytotoxicity, genotoxicity, hepatocellular carcinoma, HepG2, *Lokanatha Rasa*

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Introduction

Hepatocellular carcinoma, or HCC, ranks as the sixth most prevalent cancer and as the third leading cause of cancer-related mortality worldwide (Oh & Jun, 2023). The incidence of HCC has continued to increase, especially in areas with high prevalence of chronic hepatitis B and C virus infections (Ringelhan, McKeating, & Protzer, 2017). Chronic hepatitis, cirrhosis, oxidative stress, and persistent damage to hepatocytes are established factors in hepatocarcinogenesis (El-Serag, 2012). Despite recent advancements in chemotherapy, radiotherapy, and targeted biological therapies, the prognosis of HCC remains poor owing to drug-resistant profiles, systemic toxicities and inability of these therapies to achieve optimal therapeutic responses (Forner et al., 2018). Therefore, there is an increasing demand for novel therapeutic agents with multi-targeted biological activities and improved safety profiles.

Chronic inflammation has emerged as a key event in hepatocarcinogenesis. Inflammatory responses, being an essential criterion for chronic inflammation, facilitate tumor development. Chronic inflammation induces rapid cell division, genomic instability, oxidative DNA damage and inhibition of apoptosis, resulting in tumor development (Mantovani et al., 2008). Consequently, compounds possessing both cytotoxic and anti-inflammatory properties may offer therapeutic benefits in the management of hepatocellular carcinoma.

Ayurveda, the traditional system of medicine practiced in South Asia for centuries, describes various herbo-mineral formulations for the management of chronic diseases. *Rasa Shastra* (Alchemy), one of the specialized areas of Ayurveda pharmaceutics, mainly deals with mercurial and mineral-based preparations processed through specific purification (*Shodhana*) and incineration (*Marana*) techniques. These procedures are believed to detoxify raw materials, improve therapeutic efficacy, and enhance bioavailability. Previous scientific investigations have demonstrated that such classical pharmaceutical processes can alter the physicochemical characteristics, particle size, and biological behavior of metallic and mineral preparations, potentially contributing to their pharmacological activities.

Figure 1. LKN in capsule form of 125 mg

Lokanatha Rasa (LKN) (Figure 1) is a classical mercurial compound mentioned in *Rasendra Sara Sangrahaya;* a classical Ayurveda text (Ashok, 2003). Traditionally, LKN has been used in the management of chronic liver diseases (*Yakrit Vikara*) and conditions like splenomegaly (*Pleeha Vikara).* The formulation contains purified Mercury (*Shoditha Parada*), purified Sulphur (*Shoditha Gandhaka*), calcinated Biotite (*Abhra Bhashma*), calcinated Copper (*Thamra Bhashma*), calcinated iron (*Lauha Bhashma*) and calcinated cowries (*Kaparda Bhashma*). Betel leaf juice (juice of *Piper betle* L.) is used as the grinding agent during pharmaceutical preparation. According to *Rasendra Sara Sangrahaya,* the recommended dose for LKN is 1-2 *raththika* (125-250 mg) per day.

Assessment of cytotoxicity, anti-inflammatory activity, and genotoxicity provides important preliminary information regarding the therapeutic potential and safety profile of medicinal

formulations. Cytotoxic agents may inhibit cell proliferation, induce apoptosis, and interfere with metabolic pathways essential for tumor survival. HepG2 hepatocellular carcinoma cells are widely used in cytotoxicity studies because they retain many morphological and functional characteristics of normal hepatocytes, including stable metabolic enzyme activity (Sass et al., 1987). The MTT assay is commonly employed as a quantitative method for evaluating cell viability and metabolic activity through mitochondrial dehydrogenase-mediated reduction of MTT into formazan crystals (Mosmann, 1983). Chronic inflammation is closely associated with hepatocarcinogenesis through the release of inflammatory mediators, cytokines, and reactive oxygen species, which contribute to cellular damage and malignant transformation (Ferrero-Miliani et al., 2007). Therefore, membrane stabilization assays such as the Human Red Blood Cell (HRBC) method are commonly used to evaluate anti-inflammatory activity based on the stabilization of erythrocyte membranes under stress conditions (Mounnissamy et al., 2008). In addition, assessment of genotoxicity is particularly important for mineral-based formulations, as DNA damage and genomic instability are closely associated with carcinogenesis. The comet assay (Single Cell Gel Electrophoresis) is a sensitive technique widely used for detecting DNA strand breaks at the single-cell level and evaluating genomic damage following exposure to test substances (Tice et al., 2000; Singh et al., 1988).

Although several Ayurveda herbo-mineral formulations have been scientifically investigated for their pharmacological and anti-carcinogenic properties, evidence specifically related to the biological activities of LKN remains extremely limited. There is a lack of scientific studies evaluating its potential cytotoxic activity against hepatocellular carcinoma cells including its anti-inflammatory and genotoxic effects using established in vitro experimental models. Therefore, the present study was designed to evaluate the cytotoxic, anti-inflammatory, and genotoxic activities of LKN to generate preliminary scientific evidence supporting its traditional therapeutic applications and safety profile.

Methodology

Preparation of LKN

LKN was prepared according to the method described in the *Rasendra Sara Sangrahaya* and the Ayurveda Pharmacopeia. All raw materials were purchased from the registered drug supplier of the Faculty of Indigenous Medicine, University of Colombo, and their authenticity was confirmed using classical identification parameters. Prior to preparation, all mineral ingredients were subjected to classical *Shodhana* (purification) procedures as described in *Rasa Shastra* texts. Purification methods included grinding and filtering for *Parada* using lime water, garlic paste, and rock salt; melting and pouring for *Gandhaka* using milk and ghee; heating and quenching for *Abhra*, *Thamra*, and *Lauha* using appropriate herbal media; and steaming for *Kaparda* using lime juice. Subsequently, *Bhashma* preparations of *Abhra*, *Lauha*, *Thamra*, and *Kaparda* were prepared following classical *Marana* procedures involving repeated grinding, pellet formation, and controlled heating (*Puta*).

For the preparation of LKN, equal quantities of *Shoditha Parada* and *Shoditha Gandhaka* (50 g each) were triturated to prepare *Kajjali*. Thereafter, *Abhra Bhashma* (100 g), *Lauha Bhashma* (200

g), *Thamra Bhashma* (200 g), and *Kaparda Bhashma* (600 g) were added in the proportions mentioned in the classical text. The ingredients were thoroughly mixed and triturated repeatedly with fresh betel leaf juice (*Piper betel* L.) until a homogeneous paste was obtained. The prepared mixture was then placed inside an earthen crucible (*Sharava*) and covered with another crucible of the same size. The junction between the crucibles was sealed with mud-smeared cloth strips to prepare *Sharava Samputa*. The sealed apparatus was subjected to *Gajaputa* heating in a muffle furnace at 800°C for one hour and allowed to cool naturally. The resulting ash-colored product was finely powdered to obtain a homogeneous preparation of LKN. Three batches of LKN were prepared following the same procedure.

Ethical Clearance Statement

Ethical approval for the anti-inflammatory study of LKN was obtained from the Ethics Review Committee of *Bandaranayake* Memorial Ayurveda Research Institute/BMARI, Colombo. (ERC No -BMARI/2023/003)

Materials and Reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin-streptomycin solution, dimethyl sulfoxide (DMSO), methanol, MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Hydrogen peroxide (H_2O_2), diclofenac sodium, Alsever solution pH 6.4 (2% Dextrose, 0.8% Sodium citrate, 0.05% Citric acid, 0.42% NaCl in water), Iso saline (0.85% pH 7.2), Phosphate buffer (0.15 M, pH 7.4), Hyposaline (0.36%) and other analytical grade chemicals used in this study were purchased from Sigma-Aldrich, USA, unless otherwise specified. HepG2 cell line (ECACC General Collection; Cat No. 85011430) was used for cytotoxicity and genotoxicity studies.

Cytotoxicity Study of LKN

The cytotoxic activity of LKN against HepG2 cells was evaluated using the MTT colorimetric assay according to the method described by Cardile et al. (2004) with minor modifications. HepG2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution and maintained at 37°C in a humidified 5% CO_2 incubator (Memmert, Germany). Cells were seeded in 96-well plates (Corning, USA) at a density of 5×10^3 cells per well and incubated for 24 h at 37°C in a CO_2 incubator. LKN dissolved in DMEM was added at concentrations of 0.08, 0.1, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, and 5.0 mg/mL. Cisplatin (0.2 mg/mL) was used as the positive control, while untreated cells served as the negative control. Following a 24 h incubation period, MTT solution (5 mg/mL) was added and the plates were further incubated for 4 h. The resulting formazan crystals were dissolved using DMSO, and absorbance was measured at 540 nm using a microplate reader (BIO-RAD Model 680, USA). All experiments were performed in triplicate. Percentage inhibition of cell viability was calculated using the following formula.

% Inhibition = (OD Control – OD Test)/ OD Control x 100

Anti-inflammatory Activity of LKN

The anti-inflammatory activity of LKN was evaluated using the Human Red Blood Cell (HRBC) membrane stabilization assay according to the method described by Gandhisan et al. (1991) with minor modifications. Anonymous blood samples nearing their expiry date were obtained from the Blood Bank, Colombo and collected into heparinized tubes. Blood samples were mixed with equal volumes of Alsever solution and centrifuged at 3000 rpm for 5 min. The packed cells were washed three times with isotonic saline and a 10% v/v HRBC suspension was prepared. Methanolic extracts of LKN were prepared by macerating powdered LKN in methanol for 24 h at room temperature with intermittent shaking.

The extracts were filtered using Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (IKA-RV, 10 digital, Germany). The crude extract was stored at 4°C until further analysis. The reaction mixture contained phosphate buffer, hyposaline solution, HRBC suspension, and various concentrations of LKN extract (1.0, 0.5, 0.25, 0.125, and 0.0625 mg/mL). Diclofenac sodium was used as the standard drug, while distilled water served as the control. The mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm for 5 min. Absorbance of the supernatant was measured at 560 nm. All experiments were performed in triplicate. The percentage of HRBC membrane stabilization was calculated using the following formula.

Percentage Membrane Stabilization = 100 - Absorbance of sample x 100/Absorbance of control

Genotoxicity Study of LKN

Genotoxicity of LKN was evaluated using the comet assay (Single Cell Gel Electrophoresis) according to the method described by Tice et al. (2000) with slight modifications. HepG2 cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well and incubated overnight under standard cell culture conditions. Cells were treated with LKN at concentrations of 0.8 mg/mL, 1.6 mg/mL, and 3.2 mg/mL for 24 h. H_2O_2 was used as the positive control, while untreated cells served as the negative control.

Following treatment, cells were harvested and subjected to comet assay analysis. DNA damage was assessed by measuring comet tail length, percentage DNA in the tail, and tail moment using fluorescence microscopy. At least 50 randomly selected cells per treatment group were analyzed. All experiments were performed in triplicate. The comet score was calculated using the following formula.

Comet score = Length of comet tail / Length of comet head

Results

Organoleptic Characteristics of LKN

The formulation appeared as a fine ash-colored powder with a smooth texture, a characteristic odor, and a tasteless nature. All three batches exhibited a uniform and homogeneous appearance.

Cytotoxicity Study of LKN

HepG2 cells were treated with a range of concentrations (0.08 mg/mL – 5 mg/mL) of LKN. Table 1 presents the percentage inhibition of different concentrations of the LKN on HepG2 cells. All experiments were performed in triplicate (N = 3).

Table 1: Percentage inhibition of different concentrations of the LKN on HepG2 cells

Test concentrations (mg/mL)	% Inhibition	SD
Cisplatin (0.2)	98.7%	0.9%
0.08	-4.8%	6.6%
0.1	12.2%	1.4%
0.8	25.9%	7.1%
1.2	41.4%	14.9%
1.6	48.8%	4.0%
2	70.4%	2.8%
2.4	81.8%	8.1%
2.8	86.2%	2.1%
3.2	96.9%	1.7%
3.6	102.4%	2.9%
4	96.7%	13.0%
4.5	100.0%	7.0%
5	100.1%	7.2%

The results revealed that LKN exhibited a progressive dose-dependent cytotoxic effect against HepG2 cells (Figure 2). Approximately 50% cytotoxicity was observed at 1.6 mg/mL, corresponding to the half-maximal inhibitory concentration (IC_{50}), indicating moderate anti-carcinogenic potential. At concentrations above 2 mg/mL, more than 50% cytotoxicity was observed, indicating a strong inhibitory effect. The IC_{50} of Cisplatin (0.13 mg/mL) was significantly lower than LKN (1.6 mg/mL), indicating that LKN requires a higher concentration to achieve similar cytotoxicity.

This suggests that while LKN shows anti-carcinogenic activity, it is less potent than conventional chemotherapy drugs like Cisplatin. Beyond 2 mg/mL, the inhibition percentage increased exponentially, indicating a threshold concentration at which LKN exerts maximum cytotoxic effects.

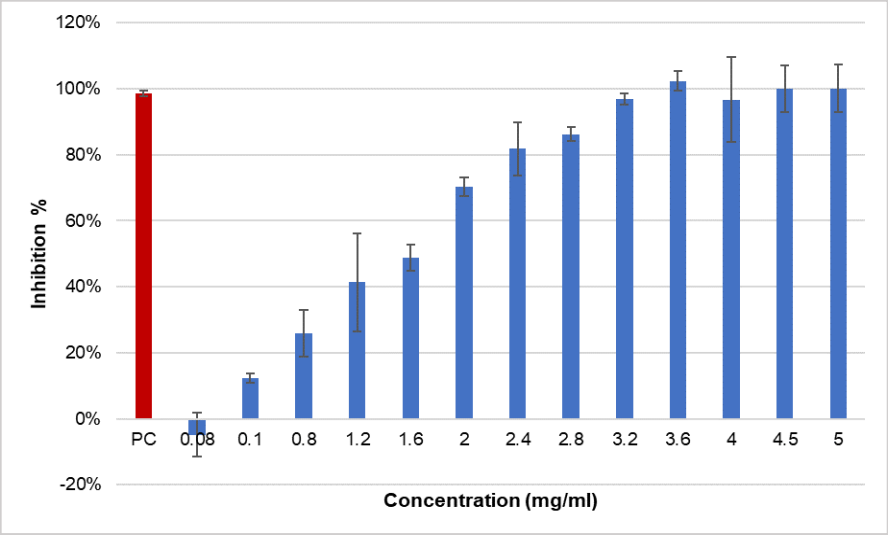

Figure 2. Effect of LKN on HepG2 viability

Anti-inflammatory Activity of LKN

Table 2: Percentage Membrane Stabilization values of different concentrations of the standard and LKN

Concentration (mg/mL)	Diclofenac (Standard) % inhibition	LKN % inhibition
1.000	69.47 ± 1.60[a]	67.01 ± 0.64[a]
0.500	64.58 ± 1.27[b]	62.54 ± 0.34[b]
0.250	59.81 ± 1.62[c]	56.36 ± 0.84[c]
0.125	49.77 ± 2.66[d]	40.19 ± 0.53[d]
0.0625	47.36 ± 1.11[e]	27.73 ± 2.45[e]

Values are expressed as mean ± SD (n = 3). Different superscript letters (a–e) indicate statistically significant differences among concentrations ($p < 0.001$).

The anti-inflammatory activity of LKN assessed by the HRBC membrane stabilization assay increased in a dose-dependent manner (n = 3) (Figure 3). Statistical analysis was performed using two-way ANOVA followed by post hoc comparison, and the differences were statistically significant ($p < 0.001$). Different superscript letters (a, b, c, d, e) indicate statistically significant differences among treatment concentrations. At the highest concentration of 1 mg/mL, the percentage of membrane stabilization activity of the standard was 69.47 ± 1.60 while in LKN it was 67.01 ± 0.64.

Therefore it is evident that, at higher concentrations, the percentage of membrane stabilization activity of LKN showed similar values comparable to diclofenac sodium, although the standard drug exhibited overall higher activity. These findings suggest that LKN possesses considerable anti-inflammatory activity under in-vitro conditions.

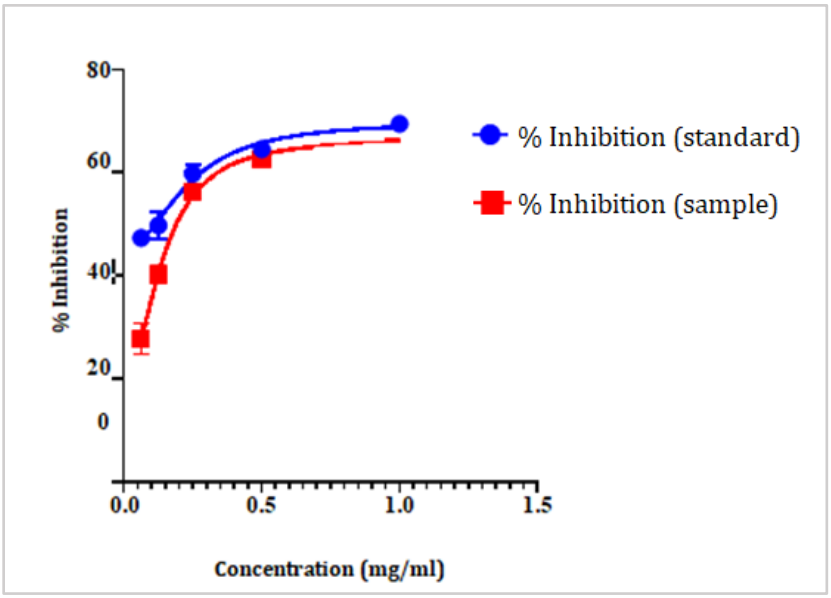

Figure 3. Anti-inflammatory effect of LKN under different concentrations

Genotoxicity Study of LKN

This comet score reflects the extent of DNA migration, with higher values indicating increased DNA damage.

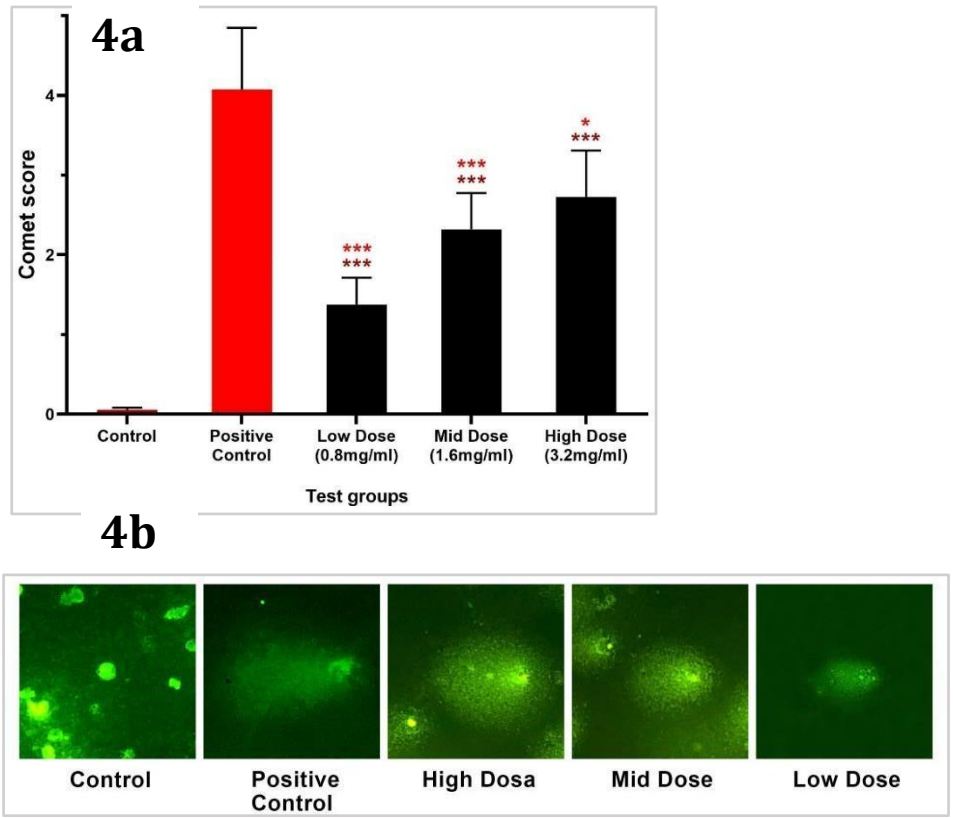

Figure 4. Evaluation of DNA damage by comet assay (4a - Comet scores, 4b - Representative comet images 60X magnification)

Figure 4a shows the Comet scores for control, LKN–treated, and positive control groups. Figure 4b shows Representative comet images (60X magnification). Cells treated with hydrogen

peroxide and medium- and high-dose LKN exhibited significant comet tail formation. Fewer comet formations were observed in the low dose group, while no comet formations were observed in the untreated control group.

Discussion

The current study offers preliminary scientific evidence for the biological activity of LKN based on the assessment of cytotoxic, anti-inflammatory, and genotoxic activities using well-established in vitro models. Although several Ayurveda herbo-mineral formulations have previously been investigated for their biological and anti-carcinogenic activities, scientific data specifically related to the anti-carcinogenic potential of LKN against HepG2 hepatocellular carcinoma cells remain limited. The results obtained suggest that LKN has concentration-dependent effects on hepatocellular carcinoma cells and possesses membrane-stabilizing and DNA-damaging activities, which may contribute to its traditional use in liver-related disorders.

HepG2 cells were originally obtained from the liver tissue of a 15-year-old Caucasian male with well-differentiated hepatocellular carcinoma. They were employed as an in vitro model because of their stable genetic background, preserved metabolic function, and ease of culturing (Bray & Moller, 2006). These cells have an adherent, epithelioid morphology, growing as a monolayer with small cell aggregates, which are widely used for drug screening and studies of the mechanisms of hepatic carcinogenesis. The MTT assay showed that LKN caused a progressive and dose-dependent reduction in the viability of HepG2 cells. At concentrations above 2 mg/mL, over 50% cytotoxicity was achieved, with a half-maximal inhibitory concentration (IC_{50}) of 1.6 mg/mL, indicating moderate anti-carcinogenic activity. In comparison, cisplatin, a standard chemotherapeutic agent, had a significantly lower IC_{50} (0.13 mg/mL), indicating greater potency. The sharp rise in inhibition at concentrations above 2 mg/mL indicates a threshold concentration beyond which LKN causes maximal cytotoxicity.

The cytotoxicity of LKN may involve several mechanisms, such as apoptosis induction, generation of oxidative stress, and inhibition of cellular metabolism. Ayurveda mineral formulations undergo classical processing methods, such as purification (*Shodhana*) and incineration (*Marana*), which may affect the physicochemical properties, decrease particle size, and increase cellular uptake, which may contribute to the observed cytotoxicity. The synergistic effect of a combination of several calcined mineral components in LKN may inhibit the growth of tumor cells and affect mitochondrial metabolism, as evidenced by the MTT assay. A multi-targeted approach is especially important in the context of complex diseases such as hepatocellular carcinoma, in which there is an interplay of metabolic dysregulation, oxidative stress, and inflammation.

Chronic inflammation is an important contributing factor in hepatocarcinogenesis, which contributes to oxidative stress, compensatory proliferation, genomic instability, and malignant transformation. Agents with anti-inflammatory activity may indirectly reduce cancer risk or development by modulating inflammation-induced cellular injury. In this experiment, the HRBC membrane stabilization assay showed that LKN has anti-inflammatory activity in a concentration-dependent manner, with values ranging from 27.73% at 0.0625 mg/mL to 67.01% at 1 mg/mL. Although diclofenac sodium demonstrated relatively higher membrane stabilization activity than LKN at lower concentrations, at the highest concentration tested (1.0 mg/mL), LKN

exhibited 67.01 ± 0.64% membrane stabilization activity, which was comparable to diclofenac sodium (69.47 ± 1.60%). Similarly, at 0.5 mg/mL, LKN showed 62.54 ± 0.34% inhibition compared to 64.58 ± 1.27% for diclofenac sodium. Two-way ANOVA showed a significant effect of both concentration and treatment on HRBC membrane stabilization activity ($p < 0.001$), indicating that the differences observed between LKN and diclofenac sodium across concentrations were statistically significant. These findings indicate that LKN possesses considerable in vitro anti-inflammatory activity and demonstrates membrane stabilization effects comparable to diclofenac sodium at higher concentrations. Membrane stabilization can be related to the ability of the formulation to protect cell membranes from stress-induced damage and may suggest the inhibition of the release of inflammatory mediators (Mounnissamy et al, 2008). This activity may complement the observed cytotoxicity by inhibiting inflammation-induced hepatocyte proliferation and oxidative stress, as indicated by its traditional use in chronic liver diseases.

The genotoxic potential of LKN was assessed using the comet assay, which assesses DNA fragmentation at the single-cell level. The data showed that LKN increased DNA migration, especially at mid and high concentrations, suggesting that LKN causes DNA damage in HepG2 cells. DNA fragmentation is commonly associated with apoptotic processes and may partially explain the observed cytotoxic effects. Induction of DNA damage is a well-acknowledged mechanism of anti-carcinogenic action, as DNA damage can interfere with cell cycle progression, replication, and induce programmed cell death. Although these results confirm the therapeutic potential of LKN, they should be viewed with caution. Excessive DNA damage may raise concerns about the safety profile of the compound, and further studies are required to assess whether the observed genotoxicity is selective for cancer cells or in normal hepatocytes (Tice et al, 2000).

This is the first study to evaluate the combined cytotoxic, anti-inflammatory and genotoxic activities of LKN against HepG2 hepatocellular carcinoma cells using in vitro experimental models. Therefore, direct comparisons with previous studies on LKN are limited. Collectively, the data from cytotoxicity, anti-inflammatory, and genotoxicity assays suggest that LKN displays multiple biological activities. LKN directly inhibits the viability of cancer cells, displays membrane-stabilizing anti-inflammatory properties, and induces DNA damage in cancer cells. The integrated activity profile suggests that the biological activity of LKN is consistent with the concept that traditional herbo-mineral compounds may act through multiple complementary mechanisms, rather than a single mechanism. Multi-target action is particularly important for the treatment of hepatocellular carcinoma, a disease that is associated with inflammation, metabolic derangement, and genomic instability. The results provide preliminary scientific support for the traditional use of LKN in liver diseases and emphasize the importance of classical Ayurveda processing of herbs to enhance biological activity. Overall, this study provides a preliminary but comprehensive evaluation of LKN's cytotoxic, anti-inflammatory and genotoxic activities, supporting its potential as a multi-target therapeutic agent in hepatocellular carcinoma and chronic liver disorders.

Conclusions

LKN has demonstrated prominent in vitro anti-carcinogenic activity against HepG2 cells, along with considerable anti-inflammatory and DNA-damaging activity. This indicates that the dose-

dependent cytotoxicity of LKN and the formation of comets are correlated with the probability of apoptosis-related mechanisms. These findings provide preliminary scientific evidence of its traditional use in liver-related diseases, thereby highlighting its potential in hepatocellular carcinoma.

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Conflict of interest statement:

The authors declare no conflict of interest.

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