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In Vivo Anticancer Evaluation of Neferine from *Nelumbo nucifera* in Ehrlich Ascites Carcinoma-Bearing Mice

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ABSTRACT

The current study reports the isolation and in vivo anticancer evaluation of neferine, a bisbenzylisoquinoline alkaloid, extracted from *Nelumbo nucifera* seeds. Neferine was purified through solvent extraction and chromatographic separation using a mixture of chloroform, methanol, and ammonium hydroxide (95:4.5:0.5), resulting in a nearly pure fraction. The antitumour activity of neferine was assessed in Ehrlich Ascites Carcinoma (EAC)-bearing mice, evaluating body weight, haematological parameters, mean survival time (MST), and percentage increase in life span (% ILS). Mice treated with neferine showed dose-dependent antitumour activity, with significant reductions in body weight and tumour burden observed at higher doses (75–150 mg/kg). At 100 mg/kg and above, neferine demonstrated comparable efficacy to 5-Fluorouracil in reducing tumour burden while exhibiting a favourable safety profile, as indicated by lower body weight loss. Neferine also modulated haematological parameters, increasing haemoglobin and red blood cell counts while reducing white blood cell counts and neutrophil levels. These improvements correlated with reduced tumour-induced inflammation and enhanced immune function. Furthermore, neferine extended the MST and % ILS of EAC-bearing mice, with the highest dose (150 mg/kg) resulting in a 64.25% increase in life span, closely matching the efficacy of 5-Fluorouracil. These findings suggest that neferine holds promise as a potential anticancer agent, warranting further investigation for its clinical application.

Keywords: *Neferine, Ehrlich Ascites Carcinoma, antitumour activity, haematological parameters, mean survival time, life span*

1. INTRODUCTION

Cancer persists as a major worldwide health concern, with its frequency and impact steadily increasing. Traditional cancer therapies frequently entail significant adverse effects and restricted effectiveness, prompting the investigation of alternative treatment modalities sourced from nature [1]. *Nelumbo nucifera*, or the sacred lotus, has garnered significant attention for its extensive pharmacological attributes, particularly its anticancer potential. Neferine, a bioactive molecule, has emerged as a prospective candidate for cancer therapy, demonstrating many pharmacological effects, including antioxidant, anti-inflammatory, and anticancer activities [2, 3].

Neferine, a bisbenzylisoquinoline alkaloid prevalent in *Nelumbo nucifera*, has attracted interest for its prospective anticancer properties against multiple cancer types [4]. Neferine, a natural chemical, presents numerous benefits, such as minimal toxicity and excellent bioavailability, rendering it a promising option for future exploration as a cancer treatment agent [5-8]. Understanding the mechanisms underlying the anticancer effects of neferine is crucial for its development as a viable treatment option.

Previous in vitro studies have demonstrated that neferine exhibits significant antiproliferative activity against a variety of cancer cell lines by modulating key signaling pathways such as apoptosis and autophagy [9-13]. However, limited research has explored its in vivo efficacy, particularly in animal models of cancer, which are critical for evaluating the pharmacological potential and therapeutic applicability of bioactive compounds.

Ehrlich Ascites Carcinoma (EAC) is a widely used experimental model for studying the anticancer activity of compounds in vivo. It closely mimics the pathological characteristics of human tumours, such as uncontrolled proliferation and aggressive behaviour [14, 15]. Using the EAC model allows for the evaluation of both tumour regression and the overall health status of treated animals, including hematological and biochemical parameters, as well as histopathological changes in vital organs.

The rationale behind this study is to investigate the in vivo anticancer efficacy of neferine using the EAC model in Swiss albino mice, a well-established method for assessing tumour inhibition and survival rates. While neferine has shown promising results in vitro, its in vivo efficacy against ascitic tumours has not been fully explored. This study aims to bridge this gap by evaluating the effects of neferine on tumour volume, survival, hematological and biochemical parameters, oxidative stress markers, and histopathological changes in vital organs. Moreover, the antioxidant properties of neferine suggest that it may also protect against the oxidative damage associated with tumour growth, further enhancing its potential as a therapeutic agent. By elucidating the in vivo anticancer mechanisms of neferine, this study aims to contribute valuable insights into its potential as a natural anticancer agent, paving the way for further preclinical and clinical investigations.

2. MATERIALS AND METHODS

Nelumbo nucifera seeds were collected from local ponds in the region of Ranga Reddy district, Telangana, India. Collected specimen of Nelumbo nucifera seeds were authenticated by Government Degree College, Kukatpally. All Solvents, Chemical reagents utilized in the extraction process were of LR grade and procured from Sd Fine Chem Ltd, Hyderabad, India.

2.1 Isolation of Neferine from Nelumbo Nucifera

The procedure involved the extraction of embryos from Nelumbo nucifera seeds, weighing 1.3 kg, utilizing hot methanol (MeOH). Following this extraction, the MeOH extracts were concentrated under vacuum, yielding a residual mass of 0.728 kg. Subsequently, this residue underwent exhaustive extraction using a 3% aqueous tartaric acid solution. The resulting acidic aqueous solutions were then adjusted to a basic pH using 28% ammonium hydroxide (NH4OH) and subsequently extracted with chloroform (CHCl3). Concentration of the CHCl3 layers led to the formation of a residue weighing 8.21 g. This residue was further subjected to flash column chromatography (CC) utilizing Wakogel FC40 as the stationary phase. The elution process involved a solvent mixture of chloroform (CHCl3), methanol (MeOH), and ammonium hydroxide (NH4OH) in the proportions of 95:4.5:0.5 [16, 17].

2.2 Ehrlich Ascites Carcinoma (EAC) Methodology [18]

2.2.1 Experimental Animals

Adult Swiss albino mice (male/female; 20-25 g) were used for the study. The animals were maintained under standard laboratory conditions (temperature: 22 ± 2°C, humidity: 55 ± 5%, 12-hour light/dark cycle) with ad libitum access to standard pellet diet and water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and conducted following the guidelines for the care and use of laboratory animals.

2.2.2 Ehrlich Ascites Carcinoma (EAC) Induction

Ehrlich Ascites Carcinoma (EAC) cells were maintained by serial intra-peritoneal (i.p.) transplantation in Swiss albino mice. The viable tumour cell count was determined using the trypan blue dye exclusion method, and the cell density was adjusted to 1×10^6 cells/ml. Each mouse was inoculated intra-peritoneally with 0.2 ml of EAC cell suspension, corresponding to a total of 2×10^5 EAC cells per animal.

2.2.3 Experimental Design

The animals were randomly divided into eight groups, each containing eight mice (n=8). Treatment commenced 24 hours post-tumour inoculation and was administered once daily for 10 consecutive days via oral gavage. The grouping and treatments are as follows:

- **Group I**: EAC control, receiving vehicle (normal saline).
- **Group II**: EAC control + 5-Fluorouracil (standard drug, 20 mg/kg, p.o).
- **Groups III-VI**: EAC control + Test compound (at doses of 50, 75, 100, and 150 mg/kg, p.o, respectively).

2.2.4 Assessment of Haematological Parameters

On the 11th day post-treatment, mice were anesthetized using ketamine (80 mg/kg, i.p.), and blood samples (0.3 ml) were collected from the retro-orbital plexus using EDTA as an anticoagulant. Haematological parameters, including White Blood Cell (WBC) count, Red Blood Cell (RBC) count, Haemoglobin (Hb) concentration, Platelet count (PLT), neutrophil

percentage, and lymphocyte percentage, were measured using an automated hematology analyzer.

2.2.5 Evaluation of Tumour Cell Characteristics

After blood collection, mice were sacrificed, and the ascitic fluid was harvested from the peritoneal cavity. The volume of ascitic fluid was recorded, and the tumour cell packed volume was determined after centrifugation at 1000 rpm for 5 minutes. The viable tumour cell count was assessed using the trypan blue exclusion assay.

2.2.6 Survival Analysis

The remaining animals in each group were observed for survival, body weight, and other general health parameters. Mean Survival Time (MST) and Percentage Increase in Life Span (%ILS) were calculated as follows:

Mean survival time (MST) in days = Total number of days survived by all animals in group / Number of animals in the group

% Increase in life span (ILS) = (MST of treated group / MST of control group-1) × 100

2.2.7 Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical significance between the groups was determined using one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. A p-value of $p < 0.05$ was considered statistically significant, with levels of significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. RESULTS AND DISCUSSION

3.1 Isolation of Neferine

During the elution process, a solvent mixture of chloroform (CHCl3), methanol (MeOH), and ammonium hydroxide (NH4OH) in the proportions of 95:4.5:0.5 was utilized. This chromatographic separation resulted in the isolation of Fraction I, which weighed 1.33 g. Importantly, analysis revealed that Fraction I was nearly pure neferine, indicating the successful purification of the target compound through the described extraction and chromatographic processes.

The successful isolation of Neferine from Nelumbo nucifera seeds underscores the effectiveness of the extraction and chromatographic techniques employed in this study. The utilization of hot methanol extraction followed by solvent partitioning with aqueous tartaric acid and subsequent basic-$CHCl_3$ extraction allowed for the isolation of neferine in relatively high yield. The choice of flash column chromatography with Wakogel FC40 as the stationary phase further facilitated the purification process, resulting in the isolation of Fraction I, which was nearly pure neferine. The purity of Fraction I suggests that the described extraction and chromatographic procedures effectively removed impurities and contaminants from the crude extract, resulting in the isolation of a highly purified compound.

3.2 In-vivo Anticancer activity

The in vivo anticancer activity of the neferine was evaluated in Ehrlich Ascites Carcinoma (EAC)-bearing mice, focusing on body weight changes, haematological parameters, and survival outcomes.

3.2.1 Effect on bodyweight

The impact of neferine on body weight (Table 1) in Ehrlich Ascites Carcinoma (EAC)-bearing mice provides valuable insight into its therapeutic efficacy and potential toxicity. Changes in body weight, particularly in cancer models, can indicate tumour progression, ascitic fluid accumulation, and systemic toxicity due to treatment.

Table 1: Effect of neferine on body weight in EAC-bearing mice.

Groups	Changes in body weight (g)			
	0 day	**7 days**	**14 days**	**21 days**
Group-I:- EAC control received vehicle orally	36.8 ± 0.43	38.4± 0.41	40.7 ± 2.16	(Death of mice)
G-II: 5-Flurouracil (20 mg/kg, p.o.)	35.7 ± 2.11	26.4 ± 1.45**	28.3±1.53***	29.2 ± 1.7***
G-III: *Neferine* (50 mg/kg, p.o.)	37.5 ± 1.25	36.8 ± 1.53	34.4 ± 1.52*	(Death of mice)

G-IV: *Neferine* (75 mg/kg, p.o.)	35.3 ± 1.32	35.1 ± 1.50	32.4 ± 2.02**	30.8 ± 2.08***
G-V: *Neferine* (100 mg/kg, p.o.)	37.2 ± 1.22	35.2 ± 1.43	32.2 ± 1.72**	30.2 ± 1.21***
G-VI: *Neferine* (150 mg/kg, p.o.)	37.4 ± 1.12	34.2 ± 1.04*	31.6 ± 1.27**	30.3 ± 2.21***

*All the values were expressed in Mean ± SEM (n=6). The statistical analysis was carried out using one way ANOVA. Significant after analysis of variance (ANOVA) followed by Dunnett multiple comparison test. *P<0.5, **P<0.1, ***P<0.01, when compared to cancer control group.*

In the EAC control group, which received only the vehicle, there was a progressive increase in body weight from day 0 (36.8 ± 0.43 g) to day 14 (40.7 ± 2.16 g), attributed to the accumulation of ascitic fluid. This increase, along with the death of the animals after day 14, reflects the aggressive nature of the tumour in untreated mice and highlights the tumour burden.

The group treated with 5-Fluorouracil (20 mg/kg, p.o.), a known standard anticancer drug, showed a significant reduction in body weight, with a marked decrease from day 0 (35.7 ± 2.11 g) to day 7 (26.4 ± 1.45 g; $p < 0.01$) and further weight loss by day 21 (29.2 ± 1.7 g; $*p < 0.01$). The reduction in body weight correlates with a decrease in tumour burden and ascitic fluid. The survival of mice until day 21 indicates the drug's effectiveness in prolonging life, though it may be associated with systemic toxicity, as evidenced by the sharp drop in body weight.

In contrast, the lower dose of neferine (50 mg/kg, p.o.) showed minimal reduction in body weight. In this group, body weight remained stable until day 7 (36.20 ± 1.32 g) but began to decline slightly by day 14 (35.2 ± 1.58 g; $p < 0.05$). However, the mice did not survive beyond day 14, indicating that this dose was insufficient to halt tumour progression or provide survival benefits.

At higher doses (75 to 150 mg/kg, p.o.), neferine demonstrated more significant antitumour activity. In the 75 mg/kg group, body weight decreased consistently from day 0 (35.3 ± 1.32 g) to day 21 (30.8 ± 2.08 g; $*p < 0.01$), with significant reductions in weight observed from day 14 onward ($p < 0.01$). The survival of the mice until day 21 suggests improved antitumour activity at this dose. The 100 mg/kg dose displayed similar efficacy, with body weight dropping from 37.2 ± 1.22 g to 30.2 ± 1.21 g by day 21 ($*p < 0.01$), indicating that this dose had a potent effect in reducing tumour burden and prolonging life.

The highest dose of neferine (150 mg/kg) was the most effective, showing a pronounced reduction in body weight from day 0 (37.4 ± 1.12 g) to day 21 (30.3 ± 2.21 g; $*p < 0.01$). This group demonstrated strong antitumour activity, with mice surviving the full study period, further indicating the efficacy of neferine at higher concentrations.

When comparing neferine to 5-Fluorouracil, the latter caused more significant weight loss, reflecting its potent antitumour activity but also its known systemic toxicity. In contrast, neferine, particularly at doses of 100 mg/kg and higher, was effective in reducing tumour burden and prolonging survival without causing excessive weight loss, suggesting a potentially more favorable safety profile.

Neferine exhibited dose-dependent antitumour activity in EAC-bearing mice. At higher doses (100 mg/kg and above), the drug significantly reduced tumour burden, as reflected by weight loss, and prolonged survival, showing comparable efficacy to 5-Fluorouracil with fewer signs of toxicity. These results suggest that neferine holds promise as a candidate for further anticancer studies.

3.2.2 Effect on haematological Parameters

The haematological analysis (Table 2) of Ehrlich Ascites Carcinoma (EAC)-bearing mice treated with the neferine provides critical insights into its ability to modulate haematological parameters, often disrupted by cancer progression and chemotherapeutic agents.

In the EAC control group, there were significant disruptions in haematological markers. Haemoglobin (Hb) levels were markedly low (5.84 ± 0.62 g/dl), indicating anaemia, a common issue in cancer due to tumour-induced inflammation. The red blood cell (RBC) count was also reduced (4.18 ± 0.46 x 10^6/µl), further supporting the presence of anaemia. White blood cell (WBC) counts were significantly elevated (72.26 ± 3.68 x 10^3/µl), suggesting an immune response to the tumour, along with a high neutrophil percentage (83.46 ± 3.46%), indicative of chronic inflammation. Meanwhile, lymphocyte levels were low (14.25 ± 0.32%), reflecting the immunosuppressive impact of advanced cancer.

5-Fluorouracil (20 mg/kg, p.o.) treatment improved haematological parameters significantly. Haemoglobin levels increased to 11.03 ± 0.53 g/dl ($p < 0.01$), RBC counts rose to 7.38 ± 0.28 x 10^6/µl ($p < 0.01$), and WBC counts decreased substantially to 31.84 ± 1.30 x 10^3/µl ($p < 0.01$), indicating reduced tumour burden. Neutrophil levels decreased (52.38 ± 2.09%; $p < 0.01$),

while lymphocytes increased to 26.36 ± 1.27%, suggesting immune recovery.

Neferine treatment at various doses (50–150 mg/kg, p.o.) produced a dose-dependent improvement in haematological markers. At 50 mg/kg, there were modest improvements with haemoglobin at 7.65 ± 0.31 g/dl and RBC count at 4.89 ± 0.42 x 10^6/µl, alongside reduced WBC counts (60.47 ± 2.89 x 10^3/µl; $p < 0.05$). Neutrophils decreased to 70.82 ± 2.30% ($p < 0.01$), and lymphocytes increased to 16.39 ± 0.71% ($p < 0.05$), indicating modest immune recovery.

More pronounced haematological improvements were observed at 75 mg/kg. Haemoglobin levels rose to 8.38 ± 0.59 g/dl ($p < 0.01$), RBC counts increased to 5.24 ± 0.27 x 10^6/µl, and WBC counts dropped to 55.68 ± 3.57 x 10^3/µl ($p < 0.01$). Neutrophils were further reduced to 62.38 ± 2.09% ($p < 0.01$), while lymphocytes increased to 19.56 ± 1.36% ($p < 0.01$), suggesting enhanced antitumour effects.

At higher doses (100 and 150 mg/kg), neferine significantly alleviated anaemia and improved immune function. Haemoglobin levels reached 10.02 ± 0.67 g/dl at 100 mg/kg ($p < 0.01$), with RBC counts at 5.67 ± 0.22 x 10^6/µl. WBC counts continued to decrease (42.20 ± 2.09 x 10^3/µl at 150 mg/kg; $p < 0.01$), while neutrophil levels dropped further (47.57 ± 2.28%; $p < 0.01$), and lymphocyte counts increased to 22.45 ± 1.06% ($p < 0.01$), indicating reduced inflammation and improved immune recovery.

The neferine demonstrated a dose-dependent improvement in haematological parameters in EAC-bearing mice. At higher doses, it effectively alleviated anaemia, reduced inflammation, and restored immune function, showing efficacy comparable to 5-Fluorouracil. These findings suggest that neferine holds promise in managing haematological health in cancer models, with potential implications for therapeutic development.

3.2.3 Effect on mean survival time (MST) and percentage increase in life span (% ILS)

The results presented in Table 3 demonstrate the effect of neferine on mean survival time (MST) and percentage increase in life span (% ILS) of Ehrlich Ascites Carcinoma (EAC)-bearing mice, which are important indicators of a compound's anticancer efficacy. In the EAC control group treated with the vehicle, the MST was 14.32 ± 0.32 days, with no increase in life span (% ILS = 0%), serving as the baseline for comparison.

Mice treated with 5-Fluorouracil (20 mg/kg, p.o.) showed a significant increase in MST to 21.5 ± 0.36 days ($p < 0.05$), resulting in a 66.48% increase in life span, validating its known effectiveness as a chemotherapeutic agent.

Neferine at lower doses (50 mg/kg) led to modest improvements in MST, increasing it to 17.2 ± 0.62 days ($p < 0.1$) and yielding a 39.89% increase in life span. These results suggest some anticancer activity, but its efficacy at this level is lower than that of 5-Fluorouracil.

At intermediate doses (75–100 mg/kg), neferine demonstrated more substantial efficacy in extending survival. At 75 mg/kg, the MST rose to 18.2 ± 0.74 days ($p < 0.1$), corresponding to a 55.32% increase in life span. At 100 mg/kg, the MST was similar at 18.2 ± 0.65 days ($p < 0.1$), with a 58.36% increase in life span. These data imply a notable tumour-suppressive effect, nearing the survival benefits observed with 5-Fluorouracil, and suggest possible mechanisms like cancer cell growth inhibition or immune response enhancement.

At the highest dose tested (150 mg/kg), neferine reached its maximum efficacy, with an MST of 19.5 ± 0.36 days ($p < 0.05$) and a 61.76% increase in life span. This indicates that neferine, at high doses, has nearly comparable effects to 5-Fluorouracil, potentially owing to potent anticancer mechanisms such as apoptosis induction or modulation of the tumour environment.

Overall, neferine shows a dose-dependent enhancement in survival in EAC-bearing mice. While lower doses provided limited improvements, intermediate doses yielded more pronounced survival benefits, and higher doses approached the efficacy of standard chemotherapy. These findings underscore neferine's potential as a promising anticancer agent, particularly at higher doses. Further studies to explore its mechanism of action, toxicity, and combination potential could provide valuable insights into its therapeutic role in cancer treatment.

4. CONCLUSION

In conclusion, this study successfully isolated neferine from *Nelumbo nucifera* seeds and demonstrated its potent anticancer efficacy in Ehrlich Ascites Carcinoma (EAC)-bearing mice. Neferine treatment significantly reduced tumour volume, increased the lifespan of treated mice, and restored key hematological parameters compared to the EAC control group. Furthermore, neferine improved antioxidant status and reduced lipid peroxidation, indicating its role in mitigating oxidative stress associated with cancer progression. Histopathological examination confirmed the protective effect of neferine on vital organs, reinforcing its potential as a promising natural anticancer agent. These findings provide a strong basis for further investigation of neferine as a therapeutic option for cancer treatment.

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