The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)
ISSN: 2791-8033

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 2025

Volume 20, Pages 19-31

ICMeHeS 2025: International Conference on Medical and Health Sciences

Cytotoxic and Antiproliferative Activities of Nymphaea Lotus and 5-Fluorouracil on Ehrlich Ascites Carcinoma Cells

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Abstract: *Nymphaea lotus* Linn (Nymphaeceae), an ubiquitious tropical water plant is a major component of herbal decoctions administered in rural Nigeria for the treatment of a wide range of diseases including cancers. However, the potency and mechanism of antiproliferative action of the plant extract against tumours are not yet established. This study was designed to investigate *in vivo* and *in vitro* the anti-tumour potency of ethanol extract of *Nymphaea lotus* (NLE) in comparison with 5-fluorouracil (Standard anti-tumour drug) in EAC-treated Swiss albino mice. 20 acclimatized adult male Swiss albino mice were inoculated with 10^6 EAC cells/mouse *intraperitoneally* and randomly divided into 4 groups of 5 mice/group. Group I served as negative control (EAC only), groups II and III received *intraperitoneal* injections of 20 and 40 mg/kg bodyweight NLE for 14 days while Group IV received 20 mg/kg fluorouracil. A fifth group of 5 mice served as the baseline control and was administered only 0.9% NaCl (Physiological saline). Tumour development was evaluated by determining the weight gain, ascitic weight gain and volume. MST and percentage ILS were also determined. DNA was extracted from EAC cells from treated animals and subjected to DNA fragmentation assay via electrophoresis. *Intraperitoneal* administration of 40 mg/kg bodyweight NLE significantly reduced the ascetic fluid volume, induced morphological changes, decreased the viability of the ascitic cells and also caused a prolongation of the lifespan of animals. *Nymphaea lotus* elicited similar pattern of responses to 5-fluorouracil which are characteristic of apoptosis.

Keywords: Ehrlich, Apoptotic, *Nymphaea lotus*, Intraperitoneal, Tumour, Fluorouracil

Introduction

Nymphaea lotus Linn (Nymphaeaceae) is a water plant generally found in Tropical and Sub-Saharan Africa. The leaves are mostly green and found floating with a spreading perianth. Compounds such as amino butanoic acid, Serine-arginine dipeptide, Tyrosine, 2-amino-7-methyl octanoic acid have been reportedly isolated from the plant (Sowemimo et al., 2007a). Its cytotoxic activity as well as telomerase inhibitory activity has also been reported (Sowemimo et al., 2007b) lending credence to its ethnomedicinal use in cancer treatment. This study was aimed at assessing the role of *Nymphaea lotus* in inhibiting Ehrlich Ascites Carcinoma in male Swiss albino mice.

Method

Materials and Methods

Trypan blue was from Bio-tech Pvt Ltd (India), 5-Fluorouracil from KOCAK FARMA (TURKEY) was used as a standard drug based on previous literature significantly enhanced the life span in EAC tumors (Muthuraman et al., 2008). All other chemicals used were of analytical grade available locally.

Plant Collection

Nymphaea lotus whole plant was collected from Osogbo, South-West, Nigeria. Plant material was identified by Mr Odewo. A voucher specimen was deposited in the University Herbarium, University of Lagos, Lagos, Nigeria with voucher number: LUH 3493.

Preparation of Aqueous Extract

The plant materials were shade dried for 3 days and pulverized into powder. Ethanol extract of the coarsely powdered material was prepared by macerating 1kg of whole plant in 1 L of distilled water for 72 hours. The marcerate was filtered and the filtrate was concentrated using the Rotary Evaporator and further concentrated to constant weight *in vacuo* using a lyotrap.

In-vitro EAC Cell Culture

Ten days after inoculation of EAT cells in the abdominal cavity of mice, the cells were isolated by needle aspiration, washed in saline, and the erythrocytes removed with a lysing solution. Ascitic tumour cell counts are done in a Cell Counting machine (Cedex, Roche) using the trypan blue dye exclusion method. Cell viability was > 95%. Tumour cell suspensions were prepared in phosphate balanced salt solution (PBS) at pH 7.4 to final concentrations of 1×10^6 viable cells ml^{-1} (Ozaslan et al., 2010).

In-vitro Cytotoxicity

In vitro cytotoxic activity was carried out using the Trypan Blue dye exclusion method. Briefly, aqueous extract of *Nymphaea lotus* in Phosphate buffered saline (1000, 100, 10, 1 and 0.1 µg/ml) were incubated with EAC cells at 37^0C. Ascitic tumour cell counts were done in a Cell Counting machine (Cedex, Roche) using the trypan blue dye exclusion method. Results were expressed as Percentage Cell viability (Saluja et al., 2011).

Animals

Adult Swiss male albino mice (26-33 g) were procured from University of Gaziantep, Turkey and used throughout the study. They were housed in prophylene cages in a controlled environment (temperature 25±2°C and 12 h dark and light cycle) with standard diet and water *ad libitum*. The animal experiments were carried out in accordance with the Institutional Protocols of Animal Care. The study was conducted after obtaining institutional animal ethic committee clearance of the University of Gaziantep, Gaziantep, Turkey.

EAC Cell Culture

Ehrlich Ascites Carcinoma cells were procured from Professor (Dr.) Mehmet Ozaslan, Department of Biology, University of Gaziantep, Turkey. They were grown by weekly *intra peritoneal* inoculation of 10^6 cells/mouse according to the method of Lawal et al. (2012). The tumour cell counts of the Ehrlich ascites cells were conducted using an automated Cell Counter (Cedex, Roche) and the trypan blue dye exclusion method.

Ehrlich ascites cells with viability above 95% were selected for the experiment. The tumour cell suspensions were prepared in phosphate balanced salt solution (PBS) at pH 7.4 to final concentrations of 1×10^6 viable cells

ml^{-1}. The mice were given *intra peritoneal* (i.p.) injection of 1×10^6 viable tumour cells per mouse in a volume of 0.2 ml according to the method of Justo et al. (2000).

In-vivo Study

Animals were inoculated with 1 x 10^6 cells/mouse on day '0' and treatment with *intra peritoneal Nymphaea lotus* extract started 24 h after inoculation, at a dose of 10, 20 and 40 mg/kg/day. The control group were treated with same volume of 0.9% sodium chloride solution. All the treatments were given for 14 days. Mortality was recorded daily. The mean survival time (MST) of each group, consisting of 5 mice was noted. The antitumor efficacy of SLE was compared with that of 5-fluorouracil (5-FU, 20 mg/kg/day, *i.p* for 14 days). The effect of NL on percentage increase in life span was calculated on the basis of mortality of the experimental mice (Sur and Ganguly, 1994). Mean survival time and Increased Life Span (% ILS) was calculated using the following equation (Mazumder et al., 1997; Gupta et al., 2000):

$$\text{MST} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\text{ILS} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100$$

Antitumor Activity

Male Swiss albino mice are divided into 6 groups (n = 5). All the groups were injected with EAC cells (0.2 ml of $1x10^6$ cells/mouse) intraperitoneally (Gupta *et al.,* 2004) except Group I. This was taken as day Zero. Twenty (24) hours after inoculation, animals start receiving daily *intra peritoneal* administration of different concentration of *Nymphaea lotus* extract.

Group I - Normal control.
Group II - Disease Control, EAC cell line ($1x10^6$ cell mouse).
Group III - EAC cell line ($1x10^6$ cells) treated with 40 mg/kg i.p NLE.
Group IV - EAC cell line ($1x10^6$ cells) treated with standard [5- flurouracil (20 mg/kg i.p.)]

After 14 days of treatment, animals from each group were sacrificed by ether anesthesia. The total number of tumour cells in the peritoneal cavity was counted by the trypan blue exclusion method (Bromberg et al., 2012) using the Cedex counter (Kavimani & Manisenthil kumar, 2000).

Ascite Volume

The ascitic fluid from the peritoneal cavity of tumour bearing mice was quantitatively isolated by peritoneal lavage after death into graduated eppendorf tubes and measured (Prakash et al., 2011).

Ascite Weight

The mice were dissected for collecting ascitic fluid from peritoneal cavity. The ascetic fluid was carefully collected with the help of 5 mL sterile syringe into pre-weighed eppendorf tubes. The ascite weight was calculated using the formula;

$$\text{Ascite Weight} = \text{Final Weight of Eppendorf} - \text{Weight of pre-weighed eppendorf}$$

Ehrlich Packed Cell Volume

The mice were carefully dissected in order to collect the ascitic fluid from peritoneal cavity. One ml of the ascite fluid of the transplantable murine tumor was carefully collected with the help of 5 mL sterile syringe. The fluid was subsequently transferred to a graduated glass centrifuge tube and centrifuged at 1000 rpm for 5 min. The fluid volume was measured. Ehrlich packed cell volume was determined using the following formula;

$$\text{Ehrlich Packed Cell volume (\%)} = \frac{\text{1- volume of fluid}}{1} \times 100$$

Blood Packed Cell Volume

One ml of blood was obtained from each mouse via left ventricular cardiac puncture and was centrifuged at 2500 rpm for 10 minutes. The Packed Cell volume was calculated by using the following formula;

$$\text{Blood PCV (\%)} = \frac{\text{1 – volume of liquid}}{1} \times 100$$

DNA Isolation from Ehrlich Ascites Carcinoma Cells

The EAC cells collected from treated and untreated animals were used for DNA fragmentation assay using the modified method of Jun-ya UEDA et al. (2002) as described briefly. Cells were washed twice in 800 µL of PBS and pelleted. Pelleted cells were lysed in 600 µL of Lysis buffer (10 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA and 0.2% Triton X-100) for 10 minutes on ice. The lysate was centrifuged at 6000 rpm for 20 mins. The supernatant was then extracted with 1000 µL of PCIAA (Phenol – chloroform - Isoamylalcohol solution, 25:24:1). The mixture was then centrifuged at 6000rpm for 20 mins and the upper layer decanted off and precipitated with 50 µL of 3M NaCl and 1000 µL of cold ethanol at -20^0C overnight. After drying, the isolated DNA was dissolved in TE buffer. Contamination by RNA was eliminated by incubation with 40 units of RNase at 37^0C for 30 minutes.

DNA Fragmentation Assay On 2% Agarose Gel

Loading buffer was added, and (fragmented) DNA electrophoresed on 2% agarose gel in TBE (40 mM Tris, 20 mM Boric acid, 1mM EDTA) at 100 V for 45 minutes and visualized by EtBr staining.

Statistical Analysis

Results are expressed as Mean ± S.E.M., SPSS package was used for data analysis and t-test was used for determining the significance ($P<0.05$) between mean values within a group.

Results and Discussion

In- vitro cytotoxicity study indicates that the aqueous extract of *Nymphaea lotus* had a dose-dependent cytotoxic effect on EAC cells *in-vitro* (Table 1).

Table 1. *In vitro* cytotoxic effect of Aqueous extracts of *Nymphaea lotus* on Ehrlich Ascites Carcinoma cells.

Concentration (µg/ml)	Mortality
1000	40.8
100	39.6
10	7.8
1	5.8
0.1	-

*Results are expressed as Mean of 3 determinations

IC_{50} value of 2691µg/ml was calculated using Probit analysis

Administration of 40 mg/kg *Nymphaea lotus* led to a significant reduction in body weight as compared to EAC tumour-bearing mice. 5-fluorouracil also caused a significant reduction in bodyweight of tumour-bearing mice (Figure 1).

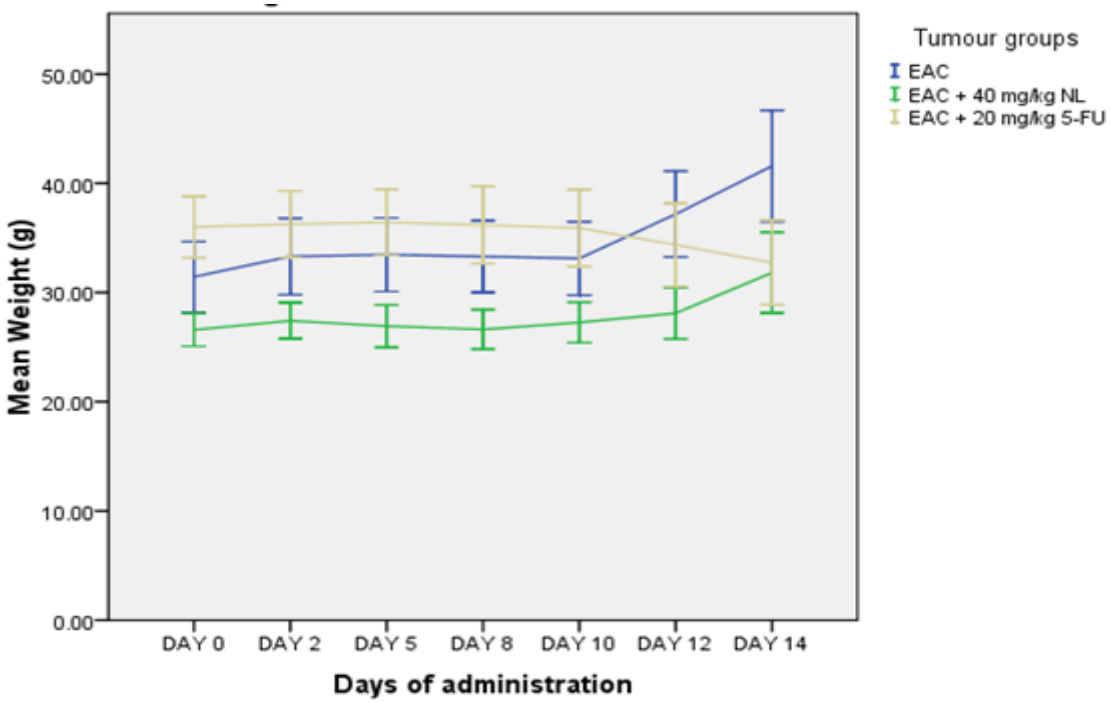

Figure 1. Effect of administration of 10 mg/kg body-weight *Nymphaea lotus* and 20 mg/kg body-weight 5-fluorouracil on the body weight of tumour bearing-mice

The effect of NLE on the survival of tumour-bearing mice is shown in Table 2. The Mean Survival time (MST) for the control group was 14.4 days whereas MST was 14.0, 14.6 and 17.4 days respectively for the groups treated with 20, 40 mg/kg bodyweight NLE and 20 mg/kg bodyweight 5-fluorouracil respectively. The percentage increase in life span of tumour-bearing mice treated with 20, 40 mg/kg NLE and 20 mg/kg 5-fluorouracil was found to be -2.7, 1.4 and 21% respectively as compared to the diseased control (Table 2).

Table 2. Mean survival time and increased life span of Ehrlich ascites-bearing mice treated with *Nymphaea lotus* extract and 5-fluorouracil*

Groups	Treatment	MST (days)	ILS (%)
1	EAC + Normal Saline	14.40 ± 0.93	-
2	EAC +20 mg/kg b.wt NL	14.00 ± 2.07	-2.7
3	EAC +40 mg/kg b.wt NL	14.60 ± 1.21	1.4
4	EAC +20 mg/kg b.wt 5-FU	17.40 ± 1.91	20.8

*Results are expressed as Mean ± Standard error of mean (S.E.M)
Values with different superscripts are significantly different from control

Reports have shown that the trypan blue assay as well as other direct cell counting methods, give more accurate results compared to other assays which take into account the metabolism of the cells (Hanaske, 1993). Saluja et al. (2011) reported that *Madhuca longifolia* leaves ethanolic extract at a concentration of 200µg/ml showed 84% activity while acetone extract caused 78% activity using the trypan blue assay. The trypan blue exclusion assay takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude the dye, trypan blue. Dead cells are selectively stained and healthy cells can be counted directly using the method of Tran et al. (2011).

According to the US NCI plant screening programme, crude extracts are generally considered to have *in vitro* cytotoxic activity if the IC_{50} value (concentration that causes a 50% cell kill) in carcinoma cells, following incubation between 48 and 72 hours, is less than 20 µg/ml, while it is less than 4 µg/ml for pure compounds (Boik et al., 2001). The high IC_{50} in this research were recorded after a short-term exposure to the extract. An increased/ long term exposure to the extract could have led to a cell kill of less than the value recorded in this study.

Table 3. Effect of *Nymphaea lotus* and 5-fluorouracil on viability of Ehrlich Ascites carcinoma cells in tumour-bearing mice*

Groups	EAC only	EAC + 20 mg/kg FU	EAC + 40 mg/kg NL
Viable Cell Count	1091 ± 11.61[a]	153.33 ± 18.49[b]	814.33 ± 267.23[a]
Dead Cell Count	413.67 ± 14.49[a]	537.33 ± 39.18[a]	4354.33 ± 291.87[b]
Total Cell Count	1505 ± 2.91[a]	690.67 ± 25.95[a]	5168.67 ± 552.61[b]
Total Cell Concentration ($\times 10^5$) (cells/ml)	423.81 ± 3.43 [a]	175.16 ± 5.14[a]	718.17 ± 76.78[b]
Viability (%)	72.46 ± 0.91[a]	22.30 ± 3.30[b]	15.07 ± 3.25[b]

*Results are expressed as Mean ± Standard error of mean (S.E.M)
Values with different superscripts are significantly different from control

Ehrlich ascites carcinoma (EAC) cells as a model in anticancer research has been proven by many authors (Clarkson & Burchenal, 1965; Prakash et al., 2011) to give accurate and reliable results as we have shown also in our previous studies. The reliability of such test lies in their ability to determine the value of any anti-cancer drug through the prolongation of experimental animals' life span in addition to changes in number and viability of the cell line itself in addition to the volume of the liquid generated by the tumour inside the peritoneal cavity (Maity et al., 1999). NL was able to elongate the life span of EAC-bearing mice. Adreani *et al.*, 1983 had suggested that an increased life span of ascites-bearing animals by 25% is considered to be an indicator of significant drug activity.

Doses of 40 mg/kg *Nymphaea lotus* when administered showed a lower ability to cause elongation of life span compared to 20 mg/kg 5-fluorouracil. 5-fluorouracil has been shown to significantly enhance the lifespan in EAC tumors (Fodstat et al., 1977) and was used as the reference drug in this study. In a previous study by Muthuraman *et al.* (2008), 100 mg/kg of *Tragia plukenetti* caused an ILS of 29.41 while fluorouracil caused an ILS of 92.88%. In this study, dose of 40 mg/kg of NL showed a lower ability to cause elongation of lifespan when compared to 5-fluoruracil (Table 2). Biswas *et al.* (2010) suggested that prevention of tumour progression by bioactive agents in medicinal plants may be responsible for the increase in lifespan observed.

In vivo, the extract was able to exert its cytotoxic effect by reducing the viable cell count when administered. This was in accordance with the findings of other researchers (Prakash et al., 2011). The increased life span reported earlier could be attributed to the reduction in the viable cell count, hence a decrease in the tumour burden. The efficacy of the extract or the reference drug is demonstrated by an ability to cause a reduction in the weight of the animals when compared to a diseased animal (Muthuraman et al., 2008). The changes in body weight monitored throughout the period of the experiment indicated a percentage decrease in the weight after administration of extract to EAC-induced animals.

The changes observed in NL-administered animals were different from those obtained when the reference drug was used. The percentage decrease in mean Ehrlich ascites weight recorded as a result of the cytotoxic effect of the extract could be responsible for the reduction in weight of animals. The standard drug, fluorouracil gave a similar response though the extract showed more potency in causing a reduction in weight. Ahmed et al. (1988) studied the interaction and *in vivo* growth inhibition of EAC cells by Jacalin. They observed that mice given an injection of EAC only had an average weight gain of 6.9 g one week after inoculation. This increase in body-weight was ascribed to accumulation of ascites fluid of EAC. However, they observed that mice receiving Jacalin (which was cytotoxic to EAC) showed a weight gain of 1.2 - 2 g after the same period.

The mean Ehrlich ascites volume, weight and Packed cell volume are used as measures of tumour growth response (Biswas et al., 2010; Prakash et al., 2011). The reduction in intraperitoneal tumour burden observed after administration of extract may be due to the cytotoxic agents present in the extract which prevent tumour progression by killing cells through the lysis of the cell membrane. The findings of this work are similar to the report of Biswas et al., 2010 in which *Dregen volubilis* fruit showed strong antitumour effect in EAC-bearing mice by causing a reduction in tumour volume, weight and viable cell count in a dose-dependent manner. In order to ensure that these malignant cells receive the nourishment they need to thrive, angiogenesis which is the formation of new blood vessels occurs (Yarbro et al., 2005). Angiogenesis has been reported to be one of the factors responsible for the accumulation of ascites fluid in the peritoneal cavity of tumour-bearing mice (Badr et al., 2011). The progressive ascitic fluid formation due to implantation of EAC causes an increasing vascular permeability due to a rise in VEGF (Fecchio et al., 1990). The reduction in EAC tumor volume may be due to a reduced expression of VEGF by the administration of extract leading to a decreased angiogenesis.

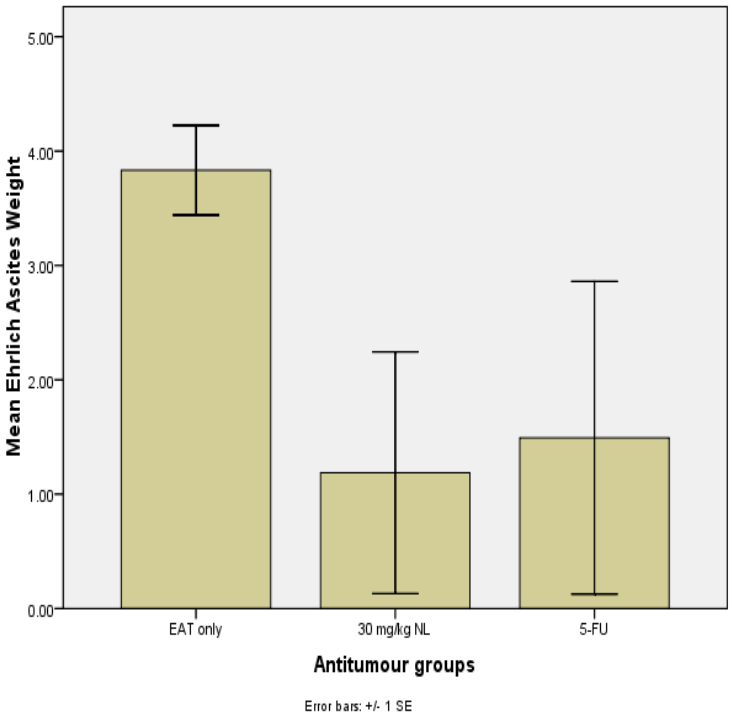

Figure 2. Effect of *Nymphaea lotus* and 5-fluorouracil on weight of Ehrlich Ascites carcinoma cells in tumour-bearing mice.
*Values are significantly different from control (P<0.05)
Keys: 5-FU (5-fluorouracil), EAC (Ehrlich ascites carcinoma). NL - *Nymphaea lotus*

Packed cell volume describes the volume that is occupied by a cell pellet after centrifugation. The % PCV value linearly correlates with the cell density. The blood packed cell volume is the volume of the cells expressed as a percentage of total blood volume (Nowak & Handford, 1994).

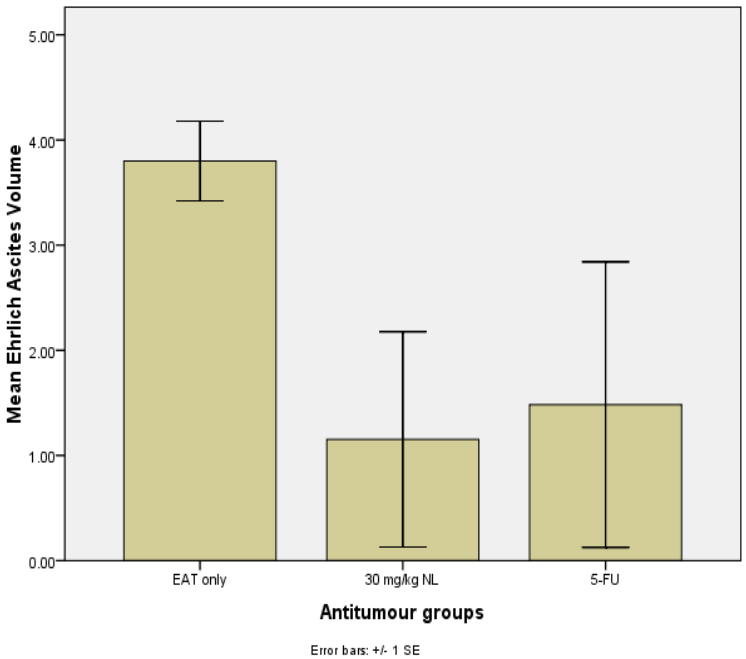

Figure 3. Effect of administration of 10 mg/kg body-weight *Nymphaea lotus* and 20 mg/kg body-weight 5-fluorouracil on Mean EAC volume of tumour bearing-mice.
*Results are expressed as Mean ± Standard error of mean (S.E.M)
Values with different superscripts are significantly different from control
EAC – Ehrlich ascites carcinoma cells, NL – *Nymphaea lotus*, 5-FU – 5-fluorouracil

The decreased Ehrlich packed cell volume observed in this experiment further shows that cytotoxic agents such as saponins present in the extract may be involved in cell lysis and hence caused a decreased percentage of viable cells. The response of EAC cells to NL *in vitro* corresponds to the behaviour *in vivo.*

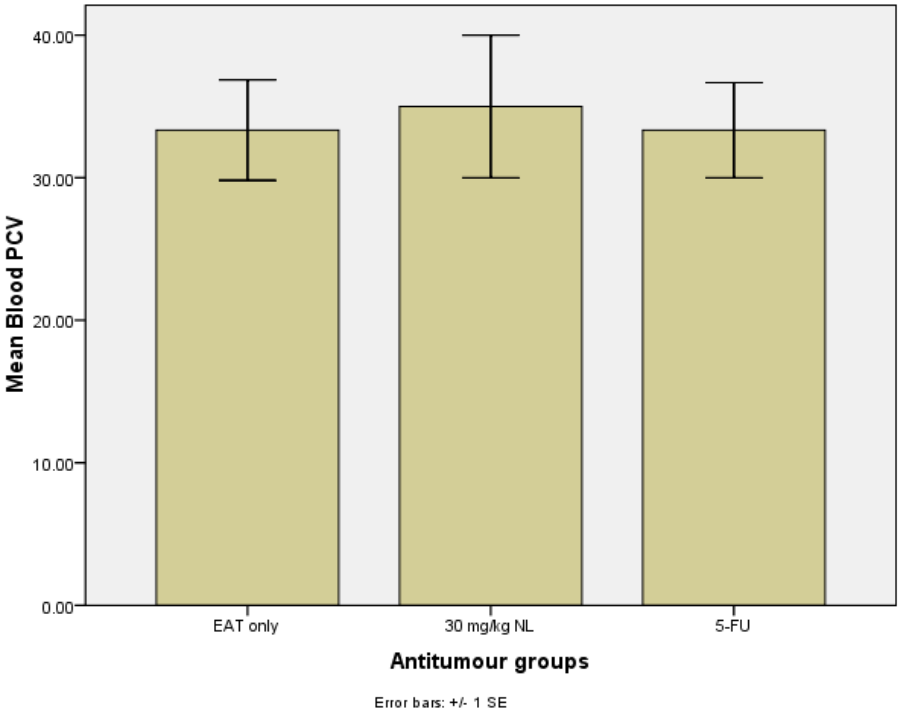

Figure 4. Effect of *Nymphaea lotus* and 5-fluorouracil on Blood PCV of tumour-bearing mice.
*Values are significantly different from control (P<0.05)
5-FU (5-fluorouracil), EAT (Ehrlich ascites carcinoma cells). NL - *Nymphaea lotus*

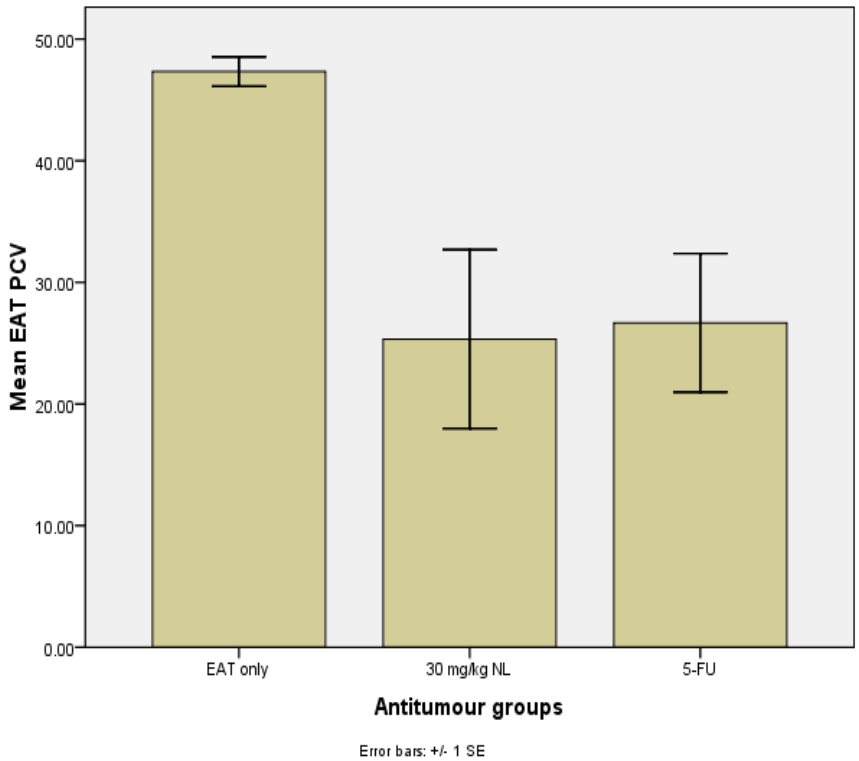

Figure 5. Effect of *Nymphaea lotus* and 5-fluorouracil on EAC PCV of Ehrlich Ascites carcinoma cells in tumour-bearing mice.
*Values are significantly different from control (P<0.05)
Keys: 5-FU (5-fluorouracil), EAT (Ehrlich ascites carcinoma cells). NL - *Nymphaea lotus*

Apoptotic cells are characterised by a number of structural and morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and the formation of apoptotic bodies (Zimmerman et al., 2001; Orienius, 2004).

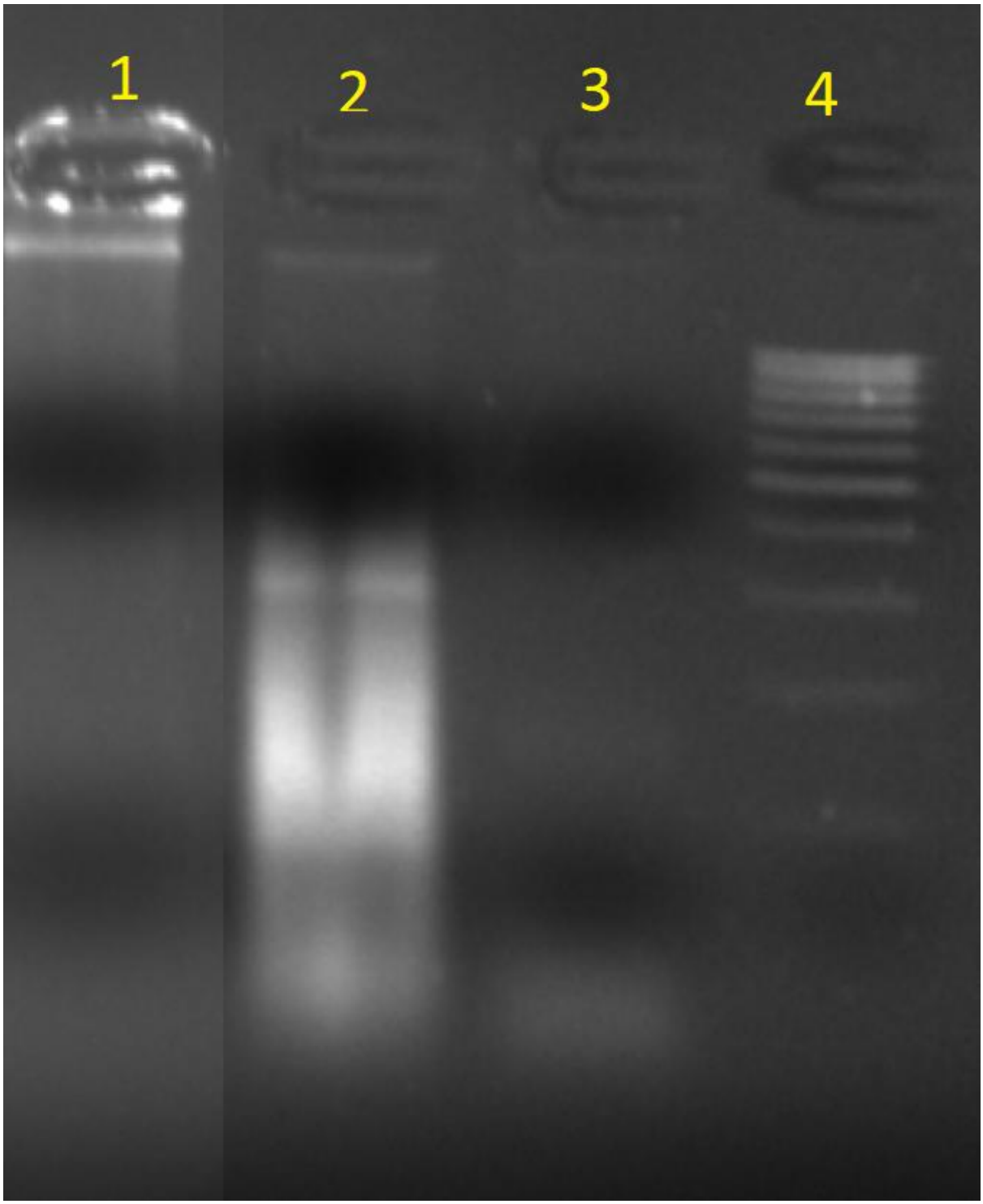

Figure 6. The aqueous extract of *Nymphaea lotus* from Osogbo, Nigeria induced DNA fragmentation in Ehrlich ascites carcinoma comparable to the effect of 5-fluorouracil. Lane 1- EAC control, Lane 2- EAC + NL extract, Lane 3- EAC + fluorouracil, Lane 4- DNA laddeer (marker).

The extract inhibited the growth of tumour cells and induced morphological changes typical of apoptosis. Results of the DNA fragmentation assay indicated that the ethanol extract of *Nymphaea lotus* induced ladder-like DNA fragmentation. The presence of this ladder has been extensively used as a marker for apoptotic cell

death (Wyllie, 1980; Nagata, 2000). However, further studies are needed for better understanding of how NL activated apoptotic cascade.

Conclusion

Nymphaea lotus showed potent cytotoxic and antiproliferative activities of *Nymphaea lotus* on Ehrlich ascites carcinoma cells which was comparable to that of 5-fluorouracil, a standard anti-cancer drug.

Recommendations

Further studies can be done to isolate and identify phytochemicals with cytotoxic and antiproliferative activity.

Scientific Ethics Declaration

* The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Conflicts of Interest

* The authors declare no conflict of interest.

Funding

* This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Acknowledgements or Notes

* This article was presented as an oral presentation at the International Conference on Medical and Health Sciences (www.icmehes.net) held in Antalya/Türkiye on November 12-15, 2025.

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To cite this article:

Lawal, R., Ozaslan, M., Adedoyin, D., & Okoye O.P. (2025). Cytotoxic and antiproliferative activities of Nymphaea lotus and 5-fluorouracil on Ehrlich ascites carcinoma cells. *The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 20,* 19-31.