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# Antitumour activity of *Annona muricata* L. leaf methanol extracts against Ehrlich Ascites Carcinoma and Dalton's Lymphoma Ascites mediated tumours in Swiss albino mice

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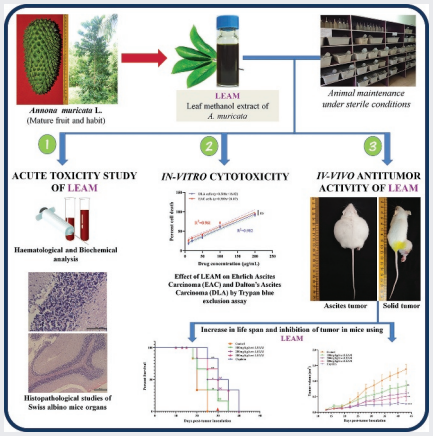
**ABSTRACT**

The use of plants as a source of sedative or treatment for cancer is reasonably widespread worldwide. *Annona muricata* Linn exhibits a vast array of medicinal and ethno-pharmaceutical benefits, attributed by different plant parts. The activity of this plant is regarded to the bio-production of secondary metabolites like alkaloids, phenols, flavonoids, and most unique group of compounds, namely, annonaceous acetogenins. Whilst this plant is gaining popularity as an anticancer treating plant, this study was undertaken to verify the plausible anticancer effect of leaf methanol extracts of *A. muricata* (LEAM). Acute toxicity study was carried to obtain safe dose in mice models using haematological, biochemical, and histological evaluations in Swiss albino mice. *In-vitro* cytotoxicity towards Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines were determined by trypan blue exclusion method. *In-vivo* antitumour activity of LEAM (100, 200, and 500mg/kg b.wt.) was evaluated using DLA induced solid carcinoma and EAC induced ascites carcinoma models and its comparison with standard drug Cisplatin. Acute toxicity studies did not exhibit significant variations in treated mice suggesting diminutive side effects of LEAM. Statistical analysis revealed the $IC_{50}$ values for DLA and EAC cell lines as 85.56 ± 5.28 and 68.07 ± 7.39 µg/mL, respectively, indicating better cytotoxic activity against EAC than DLA cells. LEAM decreased the tumour burden in dose-dependent manner. In comparison, with different concentrations tested, treatment with LEAM (200 mg/kg b.wt. and 500 mg/kg b.wt.) significantly reduced the solid tumour volume development by 58.11% and 65.70%, respectively. While lifespan was prolonged up to 51.43% in 500 mg/kg b.wt. LEAM treated ascites tumour-induced mice. This study thus indicates that LEAM possesses potent cytotoxic and antineoplastic activity and calls for more methodical safety assessments and other end-points of anti-tumourigenesis.

**Abbreviations**: **LEAM**: Leaf methanol extract of *Annona* muricata; **DLA**: Dalton's Lymphoma Ascites; **EAC**: Ehrlich Ascites Carcinoma; **$IC_{50}$**: Half maximal inhibitory concentration; **CPCSEA**: Committee for the Purpose of Control Supervision of Experiments on Animal; **IAEC**: Institutional Animal Ethics Committee; **ARRIVE**: Animal Research: Reporting *In-vivo* Experiments; **DMSO**: Dimethyl sulphoxide; **$LD_{50}$**: Lethal Dose, 50%; **SD**: Standard Deviation; **Hb**: Haemoglobin; **RBC**: Red blood cells; **WBC**: White blood cells; **HCT**: Hematocrit; **MCV**: Mean cell volume; **MCH**: Mean cell haemoglobin; **MCHC**: Mean cell haemoglobin concentration; **SALP**: Serum alkaline phosphatase; **SGPT**: Serum glutamic pyruvic transaminase; **SGOT**: Serum glutamic oxaloacetic transaminase; **ATP**: Adenosine triphosphate; **EGFR**: Epidermal Growth Factor Receptor

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## 1. Introduction

Entrenched in nature is all that is required to deal with the myriad infectious and non-infectious illnesses of ancient predominance and those of current emergence. An unprecedented spike in the number of illnesses and disorders caused by synthetic drugs has induced a switch over to conventional herbal medicine. Natural products extracted from plants; especially phytochemicals behave not only as therapeutic agents but, even more critically, in disease avoidance [1–3]. Amongst these is *Annona muricata* L. (Guanabana), belonging to family Annonaceae comprising roughly 135 genera and 2500 species. It exhibits a wide spectrum of ethnotherapeutic and efficacious properties toward cancer, accredited to various plant organs [4].

*A. muricata* has been extensively investigated for its diverse pharmacological aspects possessed by crude leaf extracts and found eminent for treating diverse maladies most critically being cancer [5,6]. According to literature, 117 isolates of secondary metabolites comprised mainly of alkaloids, annonaceous acetogenins, megastigmanes, and phenolic compounds are reported in leaves. However, this plant grown under different cultivations does not necessarily exhibit the same phytochemistry with curative benefits [7–9].

This study was undertaken due to vast research literature that brought enlightenment and motivation to conduct further clinical *in-vitro* and *in-vivo* investigations on the biological activities of methanol extracts from *A. muricata* leaves to gain an insight into its emergence as new food and pharmaceutical option. As reported, the leaves of *A. muricata* contain bioactive compounds such as annonaceous acetogenins in which annonacin was the foremost copious acetogenin isolated from leaves of *A. muricata* [10,11].

In plants, leaves are considered to account for the highest accumulation of their bioactive compounds, which are synthesized as secondary metabolites [12]. Compared with many organic solvents and aqueous media used to extract plant-based compounds, methanol seems to yield better extraction of compounds especially from the leaves [13]. It is also observed that the highest levels of alkaloids, flavonoids, phenolics, and terpenoids are extracted using methanol which is attributable to greater solubility of these bioactive compounds in methanol over certain solvents [3,14]. While major phytochemicals have been identified from *A. muricata*, the potential *in-vivo* antitumour activity of crude methanolic extracts remains rather obscure. Consequently, the present study was aimed at assessing the phytochemical potential and anticancer activity of *A. muricata* leaf methanol extracts to experimentally justify its application in mice to Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) mediated solid and ascitic tumours.

## 2. Materials and methods

### 2.1. Experimental animals

Eighty-eight adult Swiss albino mice (of both sex and weighing 20–25 g) were maintained at Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India used in the current study. Animals at the animal house facility were transferred into separate polyethylene cages. The animals were housed in a sterile setting (temperature: 24°C ± 2°C, relative humidity: 55%–65%, and 12 h dark/light rhythm) in polypropylene cages containing sterile paddy husk as bedding material with a maximum of six animals in a cage and fed standard pellets and *ad libitum* water. Animal care and maintenance conformed with recommended International Guiding Principles for Biochemical Research Involving Animals. One week before experiments, the animals were acclimatized to the laboratory environment. Protocols used in the animal model study were carried out with the prior approval of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Sanction No. 149/PO/Rc/S/99/CPCSEA) and Institutional Animal Ethics Committee (IAEC), Amala Cancer Research Centre (Sanction No. ACRC/IAEC/18(2) P-12) following Animal Research: Reporting *In-Vivo* Experiments (ARRIVE) guidelines [15].

### 2.2. Preparation of leaf extract of Annona muricata (LEAM) and dose preparation

Mature leaves of *Annona muricata* L. were procured from KOCL Research Farm, Kirbhatt, Nuvem, Goa, India (15° 18′11.27″N and 73°57′13.34″E) during the flowering stage. The plant has been identified by Dr S. Krishnan and deposited at Goa University Herbarium, Goa-India, with the voucher number AVN, AM01516. The collected materials were thoroughly washed in water, cut into small pieces and shade dried at 35°C–40°C. The dried leaves were milled to a coarse powder. Thimbles were prepared of weighed quantities (100 gms) of powder and extracted with 95% methanol by hot continuous percolation method using Soxhlet apparatus at 45°C for 12 h. Subsequently, the extract was reduced to a molten mass and dried under-reduced pressure. The solid residue was stored at 0°C–4°C for subsequent experiments. The concentrated extract (10 mg) of *A. muricata* was redissolved in 200 µL dimethyl sulphoxide (DMSO) and made up to a volume of 1 mL with PBS. The acute toxicity study of this extract was carried out on Swiss albino mice as per reported method by Lorke [16] to obtain a safe dose in mice. A test dose of 2000 mg/kg b.wt. was carried out with six animals. No, gross behavioural and mortality changes were observed at a dose level of 2000 mg/kg b. wt. suggesting the $LD_{50}$ value to be more than 2000 mg/

kg b.wt. Frequently, 1/5$^{th}$ to 1/10$^{th}$ of the lethal dose is considered for effective dose calculation. For this purpose, the limit test was performed using a dose of 500 mg/kg b.wt. that was administered to six mice (Test Group, $n = 6$) starved overnight and another group of six mice served as Control Group which was not administered leaf extract. On day 15, blood samples from both the groups were collected by direct heart puncture method and were assessed for haematological and biochemical parameters determined in a Bioanalyzer (Microlab 200) using commercial kits (Atlas Medica, UK) following standard methods [17].

The animals were then sacrificed by euthanasia under anaesthetic overdose; dissection was done and the heart, liver, kidney, and spleen were isolated and immediately stored in 10% neutral-buffered formalin at −80°C for histopathological analysis. Various organ tissues were processed for histological preparation using paraffin embedding and were sectioned at 4 µm using a microtome and stained for histological evaluations using Eosin and Hematoxylin stains [18,19].

Haematological and histological analysis of these groups did not reveal many changes indicating induction of no pathophysiological changes upon administration of a dose of 500 mg/kg b.wt. Based on these findings, LEAM doses such as 100, 200, and 500 mg/kg body weight was selected for *in-vivo* studies. Doses ranging from 10 to 200 µg/mL were used for the *in-vitro* cytotoxicity studies [20,21].

### 2.3. In-vitro cytotoxicity test and in-vivo antitumour activity of LEAM

EAC and DLA carcinoma cells were collected from tumour-bearing mice procured from the Adayar Cancer Institute, Chennai, India. DLA and EAC cells aspirated from these mice were further propagated (*in-vivo)* in mice by intraperitoneal transplantation of $1 \times 10^6$ cells suspended in 0.1 mL PBS per mouse after every 10 days to induce peritoneal tumours. Either EAC/DLA aspirated cells or tumour-induced mice were used for further experimentation to determine the antitumour properties of LEAM.

For *in-vitro* cytotoxicity study, Trypan blue dye exclusion method was used to evaluate short-term cytotoxicity. This was assessed by incubating $1 \times 10^6$ DLA and EAC cells in 1 mL PBS (pH 7.2) with initial concentrations of 10, 20, 50, 100, 200 µg/mL of LEAM at 37°C for 3 h. The percentage of cell deaths were determined by trypan blue exclusion method. Three replications of each experiment were performed and half-maximal inhibitory concentration ($IC_{50}$) of each extract was calculated [22,23].

For determination of LEAM effect on survival of ascites tumour bearing animals, five groups (6 mice/group) of mice were induced with ascites tumour by injecting $1 \times 10^6$ EAC cells/animal into the peritoneal cavity. Drug administration was initiated 24 h orally after tumour inoculation and continued for 10 consecutive days. The treatment schedule for animal groups was as follows:

Group I: EAC-bearing mice (Negative control)

Group II: EAC-bearing mice with a dose of 3.5 mg/kg b.wt. Cisplatin

Group III: EAC-bearing mice + 100 mg/kg b.wt. LEAM

Group IV: EAC-bearing mice + 200 mg/kg b.wt. LEAM

Group V: EAC-bearing mice + 500 mg/kg b.wt. LEAM.

The pattern of animal death due to tumour burden was noted daily and the mean survival time (MST) for each group was calculated [20,21]. The survival time for the treated group was subsequently compared with the control group using the following calculations:

$$\text{Percent increase in life span (\% ILS)} = \frac{MST\ of\ treated\ group - MST\ of\ control\ group}{MST\ of\ control\ group} X\ 100$$

Where MST

$$= \frac{\Sigma\ Survival\ time(days)\ of\ each\ mice\ in\ a\ group}{Total\ number\ of\ mice}$$

Five groups of mice (6 mice/group) were induced with solid tumours by subcutaneous injection of DLA cells (1 X $10^6$ cells/animal) into the right hind limb to determine the effect of LEAM on solid tumour growth. Drug administration was initiated 24 h after tumour cell inoculation orally and continued for 10 consecutive days. The treatment schedule for animal groups is as follows:

Group I: DLA-bearing mice (Negative control)

Group II: DLA-bearing mice with a dose of 3.5 mg/kg b.wt. Cisplatin

Group III: DLA-bearing mice + 100 mg/kg b.wt. LEAM

Group IV: DLA-bearing mice + 200 mg/kg b.wt. LEAM

Group V: DLA-bearing mice + 500 mg/kg b.wt. LEAM.

The radii of developing tumour were measured from 13$^{th}$ day onwards at 3 day interval using Vernier callipers and recorded up to 42 days. The tumour volume was calculated using the following formula:

$$V = 4/3\ \pi r_1^2 r_2^2$$

where $r_l$ and $r_2$ are the radii of tumour along with two directions. This was compared with the untreated control group [22,23].

### 2.4. Statistical analysis

The findings were expressed as mean ± standard deviation (SD). Statistical analyses were carried out with the aid

of GraphPad Prism Software (version 8.4.1; San Diego, CA, USA). Significance of the data was evaluated using the multiple-comparison method employing Student's t-test, One-way ANOVA and post hoc Dunnett's test. Barlett's test was also used to measure the variation relative to untreated control in various-studied groups. To analyze the comparison of survival curves, the log-rank (Mantel-Cox) test was applied. Statistical significance was taken into account at $p$-values <0.05.

## 3. Results

### 3.1. Acute oral toxicity studies of LEAM

The LEAM showed no mortality at 500 mg/kg b.wt. All animals were found to be normal with no weight changes and exhibited no gross behavioural or morphological changes till the end of the observation period of 15 days indicating that the extract was safe at 500 mg/kg b.wt.; clearly suggesting it to be nontoxic in nature.

### 3.2. Haematological evaluations

The mean haematological profiles of untreated and treated mice with LEAM are given in Table 1. The mean haemoglobin content significantly reduced ($p < 0.05$) following single-dose administration when compared to control. Relative to untreated mice, when treated with LEAM, WBC ($p < 0.01$), and platelet count ($p < 0.05$) augmented significantly. The differential blood count did not show any significant changes except lymphocyte percentage which showed a significant rise ($p < 0.01$) when treated with LEAM. However, there were no significant differences ($p > 0.05$) in RBC, HCT, MCV, MCH, and MCHC contents between control group and treated group (500 mg/kg b.wt. of LEAM).

### 3.3. Biochemical evaluations

The activities of Biochemical indices were estimated in blood serum samples as tissue function biomarkers as given in Table 1. A significant increase was found in albumin, total bilirubin ($p < 0.05$) and SALP enzyme ($p < 0.01$) activities in LEAM treated mice. However, a significant decrease was observed in Urea ($p < 0.001$), creatinine, SGOT ($p < 0.01$), and SGPT ($p < 0.05$) contents in treated group. On the other hand, the activities of total protein and globulin were not significantly altered ($p > 0.05$) when treated with LEAM (500 mg/kg b.wt.).

### 3.4. Histological evaluation

Light microscopic observations of the vital organs such as brain, kidney, liver, and spleen of the mice did not display any gross pathological abnormalities in all the control and LEAM treated (500 mg/kg b.wt.) groups for acute toxicity (Figure 1(A-H)). The control and LEAM treated groups displayed typical morphological structure in the light photomicrographs of the kidney and liver. The kidneys showed no morphological difference for the LEAM treated group. The occurrence of glomerular architecture in terms of glomeruli, distal and proximal tubules was comparable to those of control groups. There was also no interstitial and intraglomerular blockage or tubular atrophies. All nephron cells appeared normal and displayed clearly visible nuclei with no lymphocyte distortion, bruising, necrosis, or infiltration. The liver of LEAM treated animals also showed regular cellular composition,

**Table 1.** Haematological and Biochemical parameters from Swiss albino mice in the acute toxicity study by the administration of 500 mg/kg b.wt. of LEAM.

| Haematological Parameters | | | Biochemical Parameters | | |
|---|---|---|---|---|---|
| Parameters | Normal control | LEAM Treated (500 mg/kg b.wt.) | Parameters | Normal control | LEAM Treated (500 mg/kg b.wt.) |
| *Complete blood count* | | | Total bilirubin (mg/dL) | 0.28 ± 0.03 | 0.32 ± 0.03 * |
| Hb (g/dL) | 14.20 ± 0.54 | 13.37 ± 0.54 * | Total protein (g/dL) | 6.98 ± 0.50 | 7.28 ± 0.61 $^{ns}$ |
| RBC ($10^6$/μL) | 8.00 ± 0.44 | 7.63 ± 0.46 $^{ns}$ | Albumin (g/dL) | 3.70 ± 0.13 | 3.95 ± 0.23 * |
| WBC ($10^3$/μL) | 5.27 ± 0.53 | 7.00 ± 0.93 ** | Globulin (g/dL) | 3.29 ± 0.48 | 3.34 ± 0.63 $^{ns}$ |
| HCT (%) | 45.48 ± 1.36 | 44.61 ± 1.20 $^{ns}$ | Urea (mg/dL) | 50.75 ± 1.53 | 42.82 ± 1.96 * |
| MCV (fL) | 49.00 ± 3.35 | 47.36 ± 2.79 $^{ns}$ | Creatinine (mg/dL) | 0.76 ± 0.10 | 0.60 ± 0.10 ** |
| MCH (pg) | 18.08 ± 1.69 | 16.75 ± 1.10 $^{ns}$ | SALP (U/dL) | 624.28 ± 45.39 | 680.94 ± 16.56 ** |
| MCHC (g/dL) | 33.48 ± 0.67 | 33.55 ± 0.99 $^{ns}$ | SGPT (U/dL) | 56.26 ± 3.99 | 49.26 ± 5.48 * |
| Platelet count ($10^3$/μL) | 768.08 ± 60.43 | 867.14 ± 75.96 * | SGOT (U/dL) | 246.12 ± 21.30 | 203.39 ± 27.00 ** |
| *Differential count* | | | | | |
| Neutrophils (%) | 40.33 ± 4.08 | 43.67 ± 2.94 $^{ns}$ | | | |
| Lymphocytes (%) | 58.17 ± 4.36 | 64.33 ± 2.80 ** | | | |
| Eosinophils (%) | 2.17 ± 0.75 | 1.67 ± 0.52 $^{ns}$ | | | |
| Monocytes (%) | 3.17 ± 1.17 | 2.33 ± 0.52 $^{ns}$ | | | |
| Basophils (%) | 0.00 ± 0.00 | 0.00 ± 0.00 $^{ns}$ | | | |

Notes: Values represent as mean ± SD ($n$ = 6/group). Hb: Haemoglobin; RBC (Red blood cells); WBC (White blood cells); HCT (Hematocrit); MCV (Mean cell volume); MCH (Mean cell haemoglobin); MCHC (Mean cell haemoglobin concentration); SALP: Serum alkaline phosphatase; SGPT (Serum glutamic pyruvic transaminase); SGOT (Serum glutamic oxaloacetic transaminase). Data analyzed using Student's t-test where *p < 0.05, **p < 0.01, ***p < 0.001 compared to control and p > 0.05 refers to non significant difference (ns) vs. control.

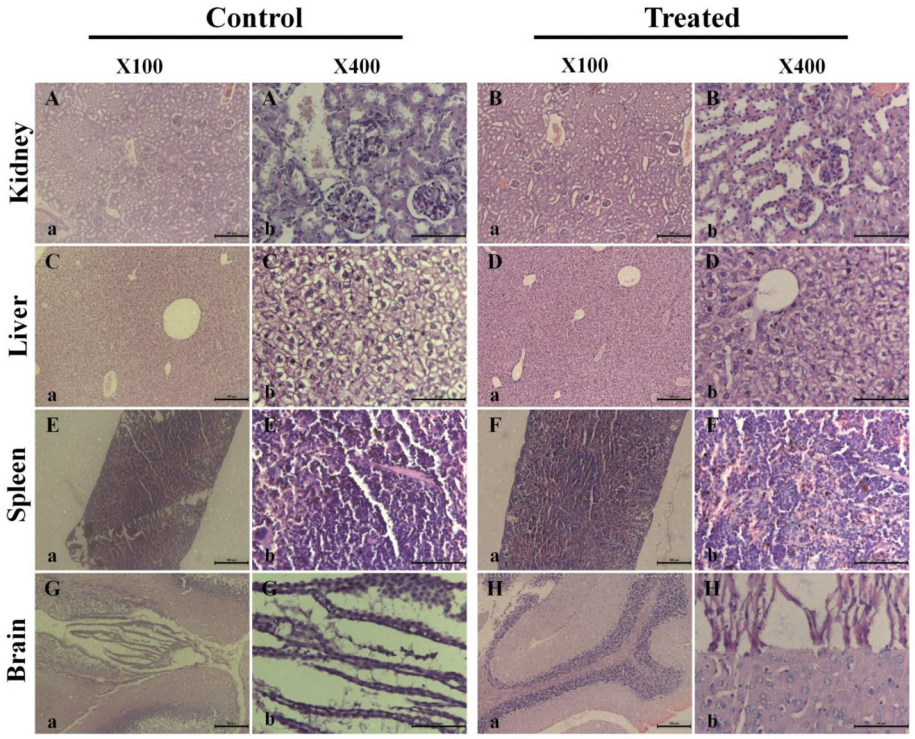


**Figure 1.** Histological sections of kidney, liver, spleen and brain of mice in acute toxicity. (A), (C), (E) and (G) represent histological sections of kidney, liver, spleen and brain of the mice of the normal control group, respectively. Whereas, (B), (D), (F), and (H) represent histological sections of kidney, liver, spleen and brain of the mice treated with 500 mg/kg b.wt. of LEAM, respectively (a: X100 and b: X400 magnification, Hematoxylin-Eosin stained).

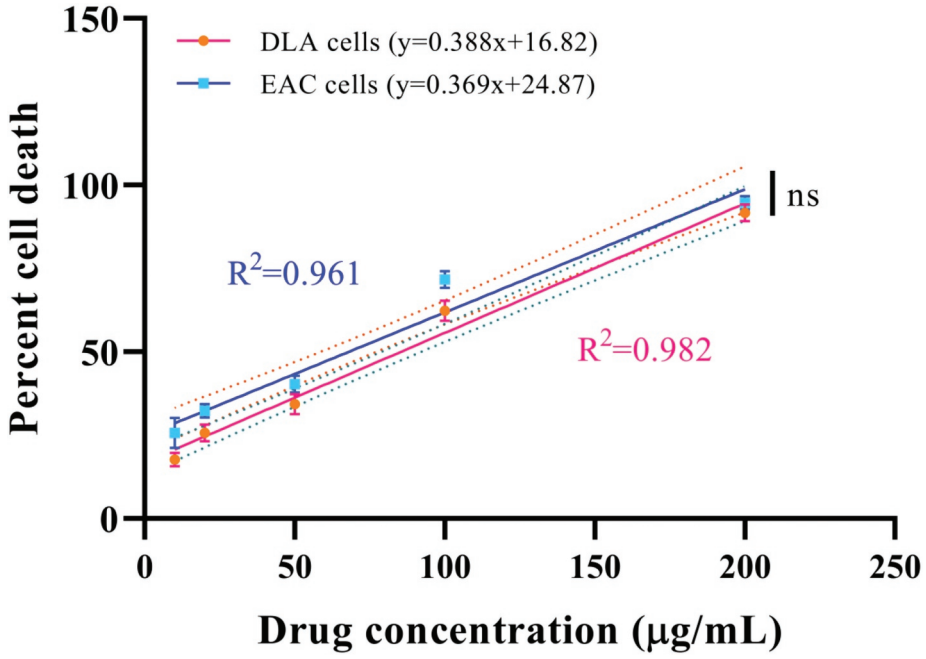


**Figure 2.** *In-vitro* cytotoxic effect of LEAM on DLA and EAC cells by trypan blue exclusion assay. Values are indicated as mean ± SD ($n$ = 3). $IC_{50}$ for DLA = 85.56 ± 5.28 µg and EAC = 68.07 ± 7.39 µg/mL.

binucleation without any distortions relative to the control group. There were also no signs of injury, necrosis, clogging, fatty acid aggregation, or hemorrhagic zones surrounding the central vein or liver sinusoids. The hepatocytes organized in cords were quite clear. Liver revealed no lyses in blood cells or infiltration of neutrophils, lymphocytes, or macrophages. Likewise, all mice showed normal structure and histology of the spleen and brain. Thus, the histopathological assessments of the selected organs did not reveal histopathies that could be attributed to the administration of LEAM to the mice.

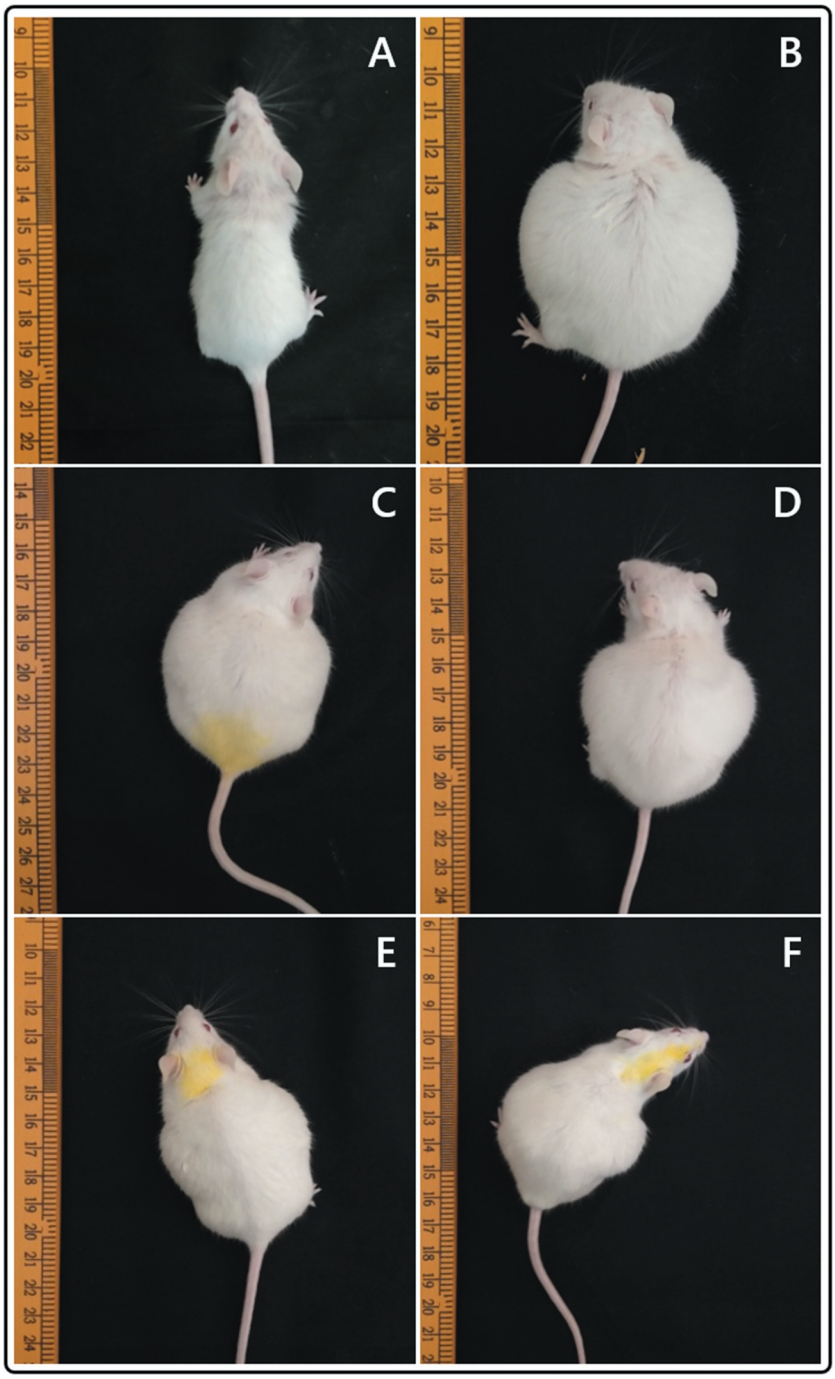


**Figure 3.** Gross appearance of mice following treatment with LEAM and Cisplatin on EAC induced ascites carcinoma bearing mice where (A) represents normal mice; (B) EAC-bearing mice; (C), (D), (E) EAC-bearing mice treated with 100, 200, and 500 mg/kg b.wt. of LEAM respectively and (F) represents EAC-bearing mice treated with single dose of Cisplatin after 24 days of tumour inoculation.

### 3.5. *In-vitro* and *in-vivo* studies of LEAM

In cytotoxicity assay, the LEAM showed excellent concentration-dependent cytotoxic potential against DLA and EAC cancer cells which is an indication of biological potency (Figure 2). The 50% inhibitory concentration values were calculated for DLA and EAC cell lines from the corresponding regression equation. The $IC_{50}$ values were 85.56 ± 5.28 and 68.07 ± 7.39 µg/mL for DLA and EAC, respectively. LEAM exhibited better cytotoxic activity against EAC than DLA cells as evident with lesser $IC_{50}$ value.

Effects of LEAM on the survival of ascites tumour-bearing animals are represented in Figure 3, Figure 5 and Table 2. Ascites tumour harbouring mice was administered with LEAM for 10 consecutive days and the days of survival were recorded. Treatments with methanolic leaf extract of *A. muricata* were found to exhibit an increase in the life span in a dose-dependent manner. With LEAM treatment, lower concentration of 100 mg/kg b.wt. exhibited 21.33%

Table 2. Effect of LEAM on survival time of EAC-bearing mice.

| Treatment | Survival time range (days) | MST (days) | ILS (%) |
|---|---|---|---|
| EAC control | 15–20 | 18.17 | - |
| EAC+100 mg/kg b.wt. LEAM | 15–30 | 21.33 | 17.43 |
| EAC+200 mg/kg b.wt. LEAM | 18–30 | 23.83 | 31.18 |
| EAC+500 mg/kg b.wt. LEAM | 20–35 | 27.50 | 51.43 |
| EAC+Cisplatin (3.5 mg/kg b. wt.) | 20–35 | 28.33 | 56.00 |

Notes: MST: Mean survival time; %ILS: Percent increase in life span

increase in life span which did not significantly alter from the control group. The administration of 200 mg/kg b.wt. of the extract on EAC induced ascites tumour showed that the plant drug was moderately efficient with 31.18% increase in life span and average life span of 23 days. Treatment of 24 h tumours with Cisplatin and 500 mg/kg b.wt. LEAM through the intraperitoneal route retarded the increase in tumour growth significantly ($p < 0.005$) by prolonging the life to 56% and 51.43%, respectively, compared to the untreated group. Consequently, this route increased the mean survival time when administered with 500 mg/kg b.wt. LEAM and Cisplatin which were found to be 27.5 and 28.3 days, respectively, compared to control having a mean life span of 18 days. All mice administered with 100 and 200 mg/kg b.wt. LEAM succumbed to death by 35$^{th}$ day whereas 500 mg/kg b.wt. and Cisplatin groups extended death up to 40 days compared to control where all mice died by 25$^{th}$ day. A higher concentration of the extract showed better efficacy compared to 100 and 200 mg/kg b.wt. of the plant drug.

Effects of LEAM on solid tumour development are given in Figure 4, Figure 6, and Table 3. The oral administration of the leaf methanol extract of *A. muricata* significantly inhibited the formation of solid tumours in a dose-dependent manner. It was observed that LEAM at 100 mg/kg b.wt. caused no significant tumour reduction compared to the untreated group. In untreated mice, the tumour volume induced by DLA cells were found to be about 15 times increased from day 0 to day 43. However, the tumour volume was found to be only 9, 8, 6, and 2 times increased in mice treated with LEAM at 100, 200, 500 mg/kg b.wt. doses, and standard Cisplatin, respectively, during these days. Significant reduction in solid tumour volume was observed for LEAM treated groups at 200 and 500 mg/kg b.wt. with 58.11% and 65.70% inhibition compared to control tumour. Whereas Cisplatin treated group showed substantially higher tumour inhibition compared to control by 86.31%. While least tumour inhibitory potential was seen at a lower dose of 100 mg/kg b.wt. LEAM with 40.36% inhibition compared to control. The higher concentration of 500 mg/kg b.wt. of LEAM was found to be more efficient in reducing the tumour volume followed by 200 mg/kg b.wt. of LEAM and 100 mg/kg LEAM during the experimental period.

## 4. Discussion

*A. muricata* is widely cultivated and possesses substantial therapeutic value and to no surprise, used in many traditions to treat multiple ailments, together with cancer primarily contributed to its cytotoxic efficacy [7,24,25]. Acute toxicity findings in the present study did not indicate significant variation in the mice behaviour which suggests no alteration in the general state of the animal after LEAM administration. Accordingly, the acute toxicity study enabled us to determine the sublethal doses of LEAM which was determined to be 100, 200, and 500 mg/kg b.wt.. Similarly, the haematological and biochemical markers showed no significant difference in all the treated mice with different doses of LEAM in comparison to control. Even after an autopsy, the histological examination of the organs showed normal architectures in the LEAM treated livers, kidneys, spleens, and brains of mice. A noticeable antitumour effect was evidenced in the present study as there was a significant reduction in tumour volume, viable tumour cell count, overall survivorship, and life expectancy of tumour harbouring mice.

Nalini and Durairaj [26] investigated the effects of hydroethanolic leaf extracts of *A. muricata* against Dalton's Lymphoma ascites-induced tumour in mice indicating that 200 and 400 mg/kg b.wt. increased life span by 26% and 33.5%, respectively. Nonetheless, when treated with 200 and 500 mg/kg b.wt. of mice, our study with methanolic extracts showed a comparatively better increase in life span by 31.18% and 51.43% of mice, respectively. This may be attributed to higher levels of alkaloids, phenolics, flavonoids, and terpenoids in methanol solvent which have higher solubility of bioactive compounds over other solvents and thus the constituents may show active synergistic effects against cancer [3,10,13,27].

*In-vitro* studies suggested that *A. muricata* leaf extract is selectively toxic against cancerous cells without harmful effects on the healthy cells [11,28,29]. Additionally, the stimulation of macrophages may facilitate the release of different types of cytokines in the peritoneal cavity which could play a role in tumour cell killing [30]. Also, bioactive compounds such as Annonaceous acetogenins, muricoreacin, and murihexocin C present in the leaves of *A. muricata* are known to show their antitumourigenic activity through the cytotoxic mode of action [31,32].

It is well documented that the cytotoxic potential of extracts from *A. muricata* may be related to the copious occurrence of annonaceous acetogenins in various plant organs. These polyketide-derived acetogenins are documented to be selectively lethal to cancer cells which act by obstructing the mitochondrial complex I leading to ATP starvation in cancer cells which have greater ATP demand unlike normal cells [29,33].

Another report by Brito *et al.* [34] noticeably discussed the antitumour and toxicity of volatile

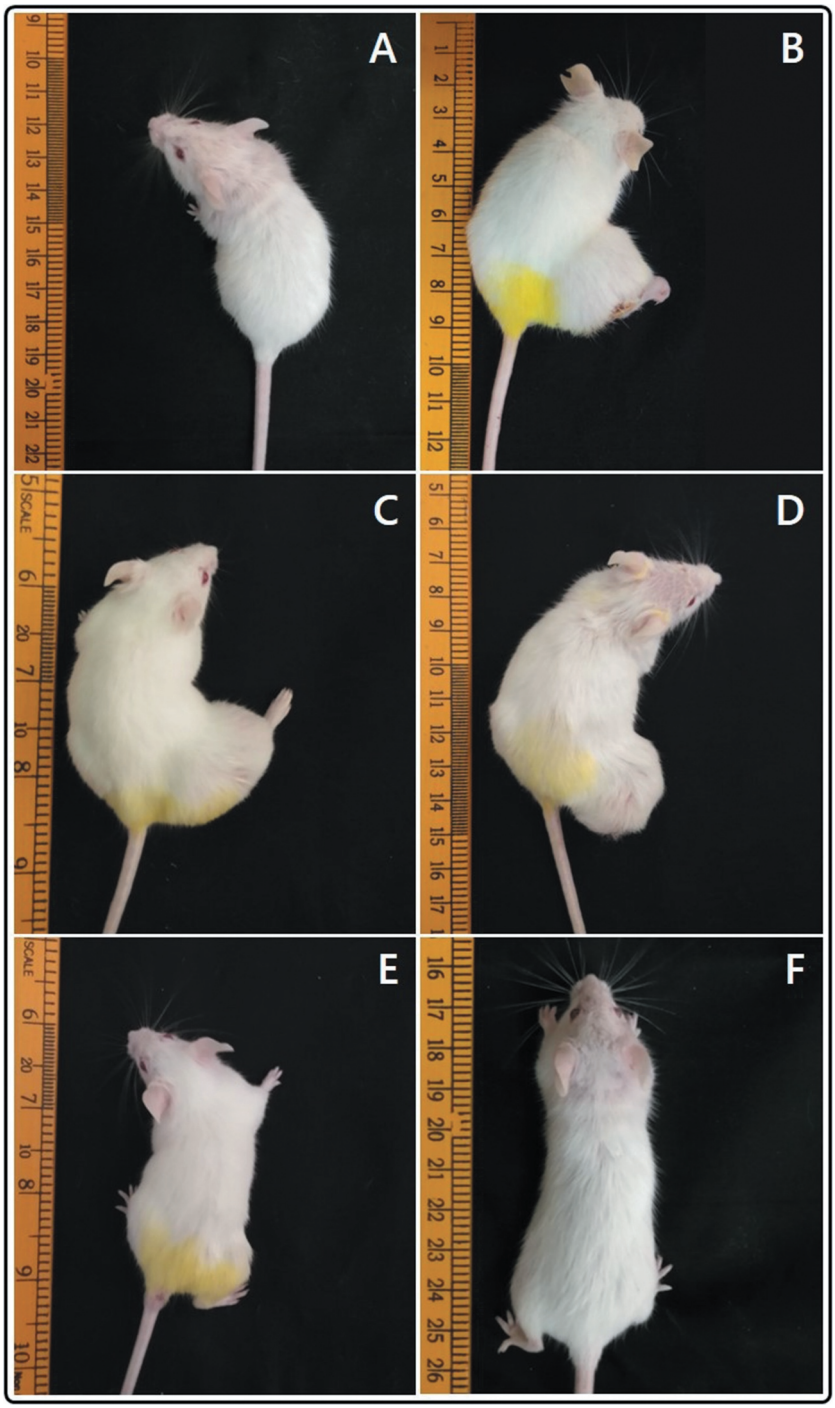


**Figure 4.** Gross appearance of mice following treatment with LEAM and Cisplatin on DLA-induced solid tumour bearing mice where (A) represents normal mice; (B) DLA-bearing mice; (C), (D), (E) DLA-bearing mice treated with 100, 200, and 500 mg/kg b. wt. of LEAM respectively and (F) represents DLA-bearing mice treated with single dose of Cisplatin after 35 days of tumour inoculation.

oil from *Annona leptopetala* leaf suggesting antitumour activity with moderate gastrointestinal toxicity. Our results corroborate these findings indicating antitumour activity without major changes in toxicity parameters evaluated. The reduction in the viability per cent and tumour cell count in treated animals as seen in this study may also be due to the selective inhibition of cancer cells via the down-regulation and inhibition of epidermal growth factor receptor (EGFR) [35,36].

Nonetheless, few investigators have reported that *A. muricata* induces cytotoxicity due to alteration in glucose metabolism resulting in unsafe metabolism obstructions. This impedance can be a target against cancer since the proliferating tumour cells have high energy demand provided by aerobic glycolysis and oxidative phosphorylation [32,37]. Some acetogenins, such as annonacin are also coupled with toxicity due to the impedances with mitochondrial performance and reduction in energy production in cells suggestive of anti-proliferative potential [7,11].

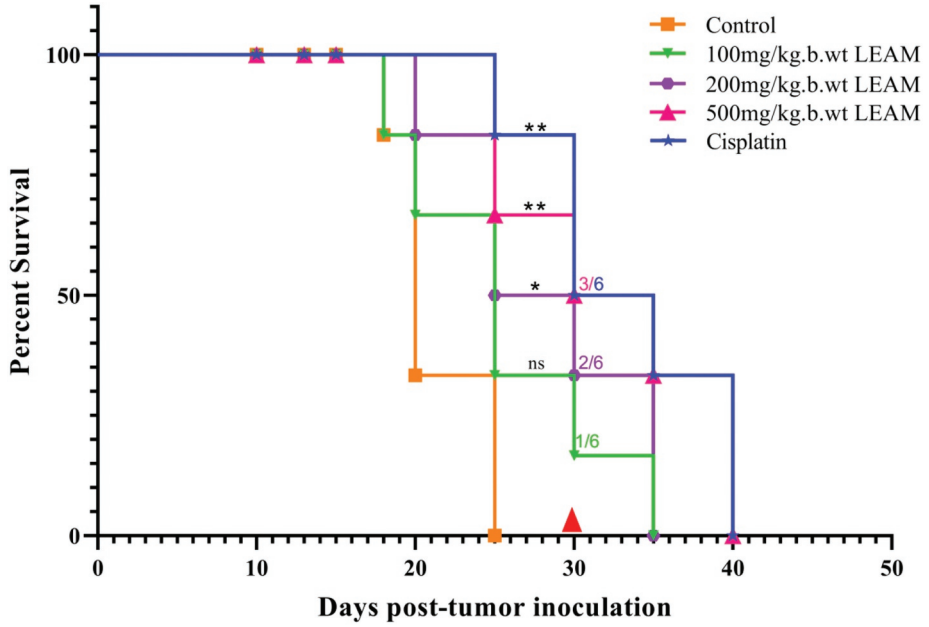


**Figure 5.** Kaplan-Meier survival curves of mice treated with LEAM (100, 200 and 500 mg/kg b.wt.) and standard drug Cisplatin (3.5 mg/kg b.wt. single dose). Results indicate that 500 mg/kg b.wt. of LEAM and standard control cisplatin significantly prolonged the survival rate of animals compared with physiological saline (Control). Survival Differences were evaluated via Log-rank (Mantel Cox) test. Statistical significance was set at a point of 95% level, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and $p > 0.05$ refers to non significant differences (ns) vs. control.

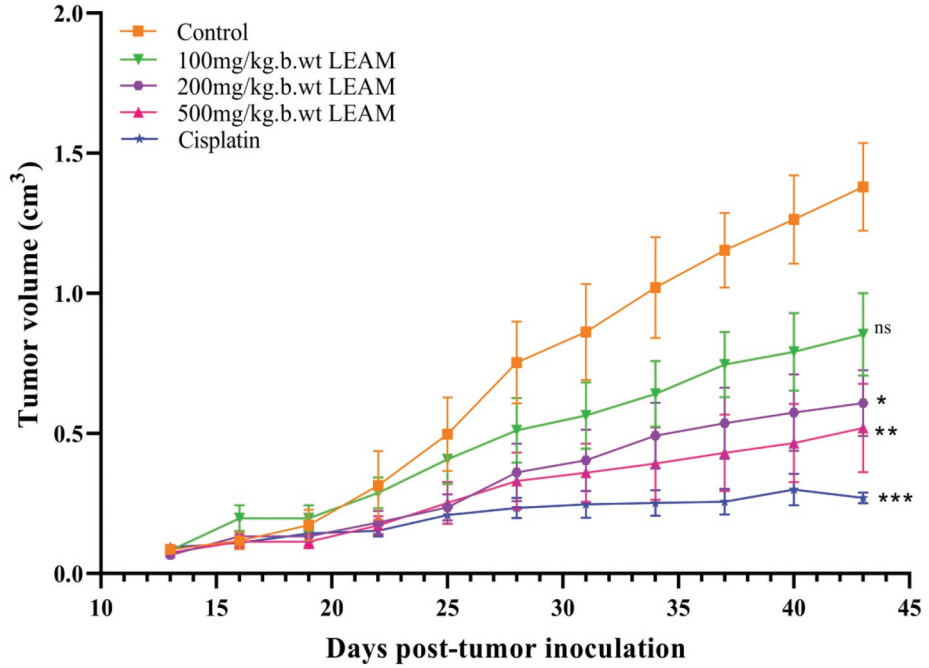


**Figure 6.** Effect of LEAM on DLA-induced solid tumour. A dose-dependent decrease in the tumour volume was observed. Values are indicated as mean ± SD ($n = 6$). Differences in tumour inhibition were assessed using one-way ANOVA with Dunnett's posthoc test and Barlett's test to compare the variance in different-studied groups. Statistical significance was set at the 95% level, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and $p > 0.05$ refers to nonsignificant difference (ns) vs. control.

While most studies performed for dose escalation to establish the efficacy of fractions in a range of diseases do not demonstrate relevant toxicity to animal models or humans, they are symptomatic that it may be safe for humans [8,38–42]. Unlike most chemotherapy, our findings suggest the efficacy of LEAM with the dose mentioned has petite side effects to host indicating as potentially useful pharmaceutical material to manage cancer.

Table 3. Effect of LEAM on DLA cell-induced solid tumour volume.

| Treatment | Tumour volume | | Δ Volume | Tumour inhibition (%) |
|---|---|---|---|---|
| | Initial | Final | | |
| DLA control | 0.086 | 1.380 | 1.294 | - |
| DLA+100 mg/kg b.wt. LEAM | 0.082 | 0.854 | 0.772 | 40.325 |
| DLA+200 mg/kg b.wt. LEAM | 0.066 | 0.608 | 0.542 | 58.111 |
| DLA+500 mg/kg b.wt. LEAM | 0.074 | 0.519 | 0.445 | 65.609 |
| DLA+Cisplatin (3.5 mg/kg b.wt.) | 0.093 | 0.270 | 0.177 | 86.305 |

## 5. Conclusion

Our study demonstrates the potency of *Annona muricata* L. methanolic leaf extracts showing *in-vitro* and *in-vivo* antitumour activity without major changes in toxicity parameters evaluated. We found a dramatic decrease in cell viability and increased protection against experimental animals from the deleterious effect of DLA/EAC induced tumour in mice exerted by LEAM in a dose-dependent manner. However, in comparison with different concentrations of leaf extract tested, 500 mg/kg b.wt. dose of LEAM regressed tumour growth and prolonged survival in mice. Hence, it is proved that *A. muricata* leaf methanol extracts possessed potent antitumour activity. This indicates that the leaf may potentially provide better bioactive compounds with substantial anti-proliferative characteristics that could be useful in primary healthcare. However, to support the above, further investigations are being carried out to elucidate the exact mechanism of action and its metabolism studies.

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## Disclosure statement

The authors declare no conflict of interest with respect to the authorship and/or the publication of this article.

## Ethical considerations

The authors assert that all procedures contributing to this work comply with the ethical standards of the national guides on care and use of laboratory animals. All experiments were carried following ARRIVE guidelines and approved by the Institutional Animal Ethics Committee at Amala Cancer Research Centre, Thrissur, Kerala–India.

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## Summary

*In-vivo* acute toxicity studies in Swiss albino mice treated with Leaf methanol extracts of *A. muricata* did not reveal significant variations signifying diminutive side effects. Furthermore, antitumour activity against Ehrlich Ascites Carcinoma and Dalton's Lymphoma Ascites mediated tumours treated with leaf extract declined the tumour burden in dose-dependent manner with lifespan prolonged up to 51.43% in 500 mg/kg b.wt *A. muricata* treated ascites tumour-induced mice. The antitumourigenic and metastatic potency determined in our analysis could therefore be linked to the synergistic activity of secondary metabolites present in leaf methanol extract of *A. muricata*.

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