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## **Antiproliferative Activity, *c-Myc* and *FGFR1* Gene Expression Profiles and Safety of *Annona muricata* Fruit Extract on Rhabdomyosarcoma and BALB/c Mice**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author NC participated in conceptualizing the study, drafting the proposal, laboratory experiments, data collection, analysis and interpretation of the findings, and drafting of the manuscript. Authors ENM, FW, and YG reviewed and edited the research proposal, guided the laboratory analysis, interpreted the findings, and reviewed the manuscript. Author WB participated in the design of the study and mentorship throughout the study. All authors read and approved the final manuscript.*

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## ABSTRACT

**Introduction:** Rhabdomyosarcoma is an aggressive solid tumour of skeletal muscles origin whose current treatment is associated with high expenses, severe side effects, drug resistance and tumour regrowth. There is a need to develop safer and more effective chemotherapeutic agents. *Annona muricata* is one of the widely used plants in treating various diseases due to its reported effectiveness. However, there is a dearth of scientific information regarding the efficacy of *Annona muricata* on rhabdomyosarcoma and its safety. This study aimed to evaluate the effects of *Annona muricata* ethanolic fruit extract on the antiproliferative activity and gene expression in RD cell line, including its biosafety in BALB/c mice.

**Materials and Methods:** The resazurin metabolic assay was used to assess the antiproliferative and cytotoxic activities of *Annona muricata* ethanolic fruit extract on RD and Vero cells. Quantitative real-time polymerase chain reaction was used to assess the gene expression profiles on *c-Myc* and *FGFR1* genes. To evaluate the safety of the *Annona muricata* ethanolic fruit extract, an acute oral toxicity study was conducted on BALB/c mice.

**Results:** *Annona muricata* ethanolic fruit extract significantly inhibited the growth of RD cells in a concentration and time-dependent manner while being highly selective on the Vero cells (selectivity index of 6.10 at 72h) compared to a reference cancer drug, doxorubicin (Selectivity index of 1.38 at 72hr). The *c-Myc* and *FGFR1* genes were under expressed in RD cells treated with *Annona muricata* ethanolic fruit extract with (3.4 and 6.1 fold), respectively, compared to untreated cells. Acute oral toxicity studies revealed no significant difference ( $p \geq 0.05$ ) between the treated mice and the control group, indicating the safety of the fruit extract.

**Conclusion:** *Annona muricata* ethanolic fruit extract can serve as effective and safe anticancer agents against rhabdomyosarcoma and further develop into standard drugs. Non-human primate studies need to be undertaken to step towards the clinical utilization of the *Annona muricata* ethanolic fruit extract in the management of rhabdomyosarcoma.

**Keywords:** Rhabdomyosarcoma; *Annona muricata*; *c-Myc* gene; *FGFR1* gene; RD cell line.

## DEFINITIONS, ACRONYMS, ABBREVIATIONS

RD : Human rhabdomyosarcoma cell line  
 ALT : Alanine aminotransferase  
 AST : Aspartate aminotransferase  
 RBCs : Red blood cells  
 HCT : Hematocrit  
 MCH : Mean Cell Hemoglobin Content  
 MCV : Mean Cell Volume  
 HB : Hemoglobin  
 WBCs : White blood cells  
 SI : Selectivity index

## 1. INTRODUCTION

Cancer is among the leading causes of morbidity and mortality worldwide [1]. An estimated 18.1 million people in 2018 were diagnosed with cancer globally, and 9.6 million succumbed to it [2]. World Health Organization (WHO) projects the annual cancer morbidity rate to double in the near future to 29 million new cases by 2040, with developing countries bearing a tremendous burden [3]. Therefore, there is an urgent need to unveil new remedies to combat this disease.

Rhabdomyosarcoma is an aggressive malignancy that originates from skeletal muscle progenitor cells [4]. It affects children and adolescents less than twenty years, with an annual prevalence of 4.5 cases per 1 million children [5]. In Africa, incidence rates of rhabdomyosarcoma vary considerably from 6-35.7 %, with East Africa having the highest rate [6]. Rhabdomyosarcoma patients have a survival rate of about 60% due to a combination of surgery, radiotherapy and chemotherapy [7,8]. However, in recent times there has been minimal improvement in the clinical oncological outcome of patients [8,9]. The high cost of drugs, side effects, drug resistance, severe toxicity, and tumor regrowth represent the most common treatment failure in patients with [10]. Considering the health effects of the current treatment strategies used in the treatment of rhabdomyosarcoma, there is a need to find safer and more effective, and cost-effective readily available chemotherapeutic agents.

Among the hallmarks of cancer, metastasis, cell proliferation, differentiation, and angiogenesis are critical in tumorigenesis [11,12]. Recent molecular and genetic analysis has shown that

deregulation of *c-Myc* and *FGFR1* genes are linked to tumor aggression, resistance to therapy, and poor clinical outcome in patients with rhabdomyosarcoma [13–15]. *FGFR1* gene belongs to a group of growth factors receptor tyrosine kinases which regulate fundamental cellular processes such as; angiogenesis, wound healing, adipogenesis, and neurogenesis [13,16]. *c-Myc* gene is a proto-oncogene involved in several biological mechanisms such as cell proliferation, differentiation, and apoptosis [14]. Therefore, finding a chemotherapeutic agent that can inhibit *c-Myc* and *FGFR1* gene expression levels in rhabdomyosarcoma may help develop an efficacious chemotherapeutic agent to control the disease.

For centuries medicinal plants have been used to treat cancer and other diseases. Hence they have been proposed as an alternate medicament source [17]. Medicinal plants are a source of bioactive compounds such as alkaloids, phenols, flavonoids, terpenes, among others [18]. These bioactive compounds have been shown to have selectively possessed therapeutic effects of anti-metastatic antiproliferative, pro-apoptotic, and anti-angiogenic therapeutic effects in cancer cells [18,19]. Despite the widespread global utilization of medicinal plants in drug discovery and development, a few have been scientifically investigated for their safety and efficacy [20]. Hence screening for potential pharmacological toxicity effects of medicinal plants is vitally important.

*Annona muricata*, also known as Soursop in English or Matomoko in Kenya [21], is a medicinal plant widely distributed in Africa's tropical regions [22]. Leaves, roots, fruits, and seeds of *Annona muricata* are traditionally utilized to treat various diseases such as diabetes, hypertension, skin rashes, stomach pain, malaria, cancer, diabetes, hypertension, and respiratory illness [23–25]. The predominant phytochemicals such as acerogenins, alkaloids, and phenolic provide the plant with various biochemical properties and health benefits such as anti-cancerous [26], anti-bacterial [22], anti-viral, antioxidant, and anti-fungal activities [23]. Identified 3-Dimethylthiourea and Quinoline compounds from extracts of fruits of *Annona muricata* showed significant anticancer activity against selected adenocarcinomas [27,28].

Although there is evidence of *Annona muricata* fruit extract having anticancer activity against various cancer cells, there is sparse

information regarding its activity on rhabdomyosarcoma. The current study aimed to investigate the effects of *Annona muricata* ethanolic fruit extract on the proliferative activity and gene expression in RD cell line, including its biosafety in BALB/c mice.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

DMEM, L-Glutamine, Fetal Bovine Serum (FBS), Streptomycin/Penicillin, Phosphate Buffered Saline (PBS), and Trypsin-EDTA were purchased from Sigma Aldrich (Germany). Resazurin and doxorubicin were purchased from Solarbio (China).

### 2.2 Plant Collection and Authentication

Mature *Annona muricata* fruits were collected in September from Machakos, Masinga County, Kenya: coordinates 00 53'21" S, 37'35'40" E, altitude 1046 m. The fruits were identified and authenticated at Jomo Kenyatta University of Agriculture and Technology Botanical Herbarium by Mr Muchuku John Kamau. A voucher specimen was deposited in the herbarium under the collection number NC-JKUATBH/001/AM/A-2020.

### 2.3 Preparation of Plant Material

The fruits of *Annona muricata* were cleaned with distilled water, cut into minute pieces using a sharp knife, and dried at room temperature for two weeks at the Pan Africa University of Institute Science and Basic Technology laboratory. The dried fruits were then pulverized into a powder, then weighed and stored in a closed container at 4°C until use.

### 2.4 Preparation of Ethanolic Fruit Extract

The solvent extraction technique as previously described [27] was employed with slight modification. Briefly, about 500g of *Annona muricata* fruit powder was macerated into 1500ml of absolute ethanol for three days. The ethanolic extract was filtered using Whatmans' filter paper grade No. 1. The filtrate was then concentrated under pressure at 45°C in a rotary evaporator (LabTech®) to obtain the concentrated extract. The final mass of the extract was recorded and used to calculate the percentage yield. The residues from the extracts were collected and stored in a closed container at 4°C before use.

## 2.5 Cell lines and Culturing Conditions

The cell lines utilized in this research were RD (CCL-136TM) and Vero E6 (CRL-1586TM). Supplementary Fig. 1 shows the photomicrographs of the cell lines. RD is the human rhabdomyosarcoma cancer cell line [29], while the Vero E6 cell line are normal kidney monkey cells, which are used as the normal cell line to assess the effects of chemicals or other substances on mammalian cells [30]. The cell lines were purchased from the American Type Culture Collection (ATCC) and sub-cultured at the Center for Virus Research, Kenya Medical Research Institute (KEMRI). The two cell lines were cultured separately in Dulbecco's Modified Eagle's Medium (DMEM) T-75flasks. Supplemented with L-glutamine, 10% FBS and 1% streptomycin/penicillin and maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub>. At 80% confluence, the cells were harvested using trypsin-EDTA solution and subculture onto 96 well cell culture plates. All culturing works were done with sterile techniques in a biohazard safety cabinet class II.

## 2.6 Assessment of Cell Viability Using Resazurin Metabolic Assay

The resazurin metabolic assay was used to assess the effects of *Annona muricata* ethanolic fruit extract on the viability and cytotoxicity of the cells. The assay is based on indicator dye (resazurin) reduced to resorufin by variable cells. Non-viable cells do not produce any fluorescent signal due to rapidly lose the metabolic capacity to reduce resazurin [31]. Briefly, cell lines at 80% confluence were washed with hank's balance salt solution and detached by trypsinization. The number of viable cells was counted using the hemocytometer after being stained with trypan blue. The trypsinized RD and Vero cells with a cell density of  $10.0 \times 10^4$  were seeded onto flat-bottomed, 96-well plates and incubated for 24 hrs at 37°C in a 5% CO<sub>2</sub> incubator to allow attachment overnight. Stock solution (10mg/ml) was prepared in 0.5% of DMSO in media. After 24hr, seeding media was aspirated from the plates with the cultured cells. Series of working Concentrations (200 - 6.25 ug/ml) were prepared by dilution from the stock and then added to the cultured cells. Doxorubicin was used as positive control drug for cancer. The cells with treatments were then returned to the incubator for 24hr, 48hr, and 72hr time points. After the specified times of exposing the cells to the treatments,

3mg of resazurin dye was weighed and dissolved into 20ml of filter-sterilized PBS, making a concentration of 0.15mg/ml. Next, 20ul of resazurin dye was added to the treated cells and incubated for 4-hours. Plates were then read on Infinite M1000, Tecan (plate reader) using the absorbance of 570nm-600nm. All treatments were done in triplicates (n = 3). The 50% inhibition concentrations (IC<sub>50</sub>) for cancer cells and the 50% cytotoxicity concentration (CC<sub>50</sub>) for normal cells were calculated and analyzed using nonlinear regression analysis, and the dose-response curve was plotted (Graph Pad Prism software version 8.4.3). The values were analyzed using One-way ANOVA followed by Tukey's test to determine statistical significance in time exposure among the treatments, and P-value was set at 0.05.

## 2.7 RNA Extraction and cDNA Synthesis

RD cells at 80% confluence in T-75 flasks were exposed to IC<sub>50</sub> treatments at 24, 48, and 72 hours. RNA was extracted from samples utilizing the DirectZol kit (Zymo Research, USA) following the manufacturer's instructions. Briefly, the samples were lysed in TRI reagent and directly added to the Zymo-Spin™ II Column, followed by centrifugation and washing. The samples were subsequently treated with DNase 1 to avoid genomic DNA contamination before RNA elution. The purity and integrity of RNA extracted from RD cells were assessed by nanodrop spectrophotometry (260nm and 280nm) (ThermoFisher) and visual inspection of the three rRNAs (28,18and 5s) on 1% agarose gel electrophoresis. The RNA concentration in all samples was adjusted to a baseline of 100ng/μl to compare gene expression measurements. Complementary DNA (cDNA) synthesis was carried out using FIRE Script RT cDNA synthesis kit (Solis BioDyne, Estonia), following the manufacturer's protocol. A final volume of 20μl reaction mixture comprised: 10μl of template RNA (100ng/μl), 1μl oligo (dT) primer (100μM), 0.5μl dNTP mix (20mM), 2μl 10x RT reaction buffer with DTT, 1μl FIRE Script RT, 0.5μl RNase inhibitor (40U/ μl) and 5 μl nuclease-free water. The reverse transcription process was carried out in an ABI 7500 PCR machine (Applied Biosystems) under 55°C for 60 minutes for reverse transcription and 85°C for 5 minutes for enzyme inactivation.

## 2.8 Primers Designing for Targeting Genes

Quantitative real-time polymerase chain reaction was performed to determine the expression levels of *c-MYC* and *FGFR1* in RD cells when treated with doxorubicin and *Annona muricata* ethanolic fruit extract. *B-actin* served as the housekeeping gene. The primers were designed utilizing the National Center for Biotechnology Information (NCBI) Primer Blast tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>). The primer sizes ranged from 70-250 bps and had a 40-60 % GC content and self-complementarity  $\leq 2$ . The online Sequence Manipulation Suite bioinformatics tool (SMS) was used to evaluate the binding specificities of the primers. Details of the primers are shown in Table 1.

## 2.9 Quantitative Real-time Polymerase Chain Reaction

The Quantitative real-time polymerase chain reaction was used to evaluate the effect of doxorubicin and *Annona muricata* ethanolic fruit extract on gene expression profiles of *c-Myc* and *FGFR1* genes.  $\beta$ -Actin was used as the housekeeping gene. The Quantitative real-time polymerase chain reaction (in triplicates) was set up with 5x HOT FIREPol® EvaGreen® (Solis-BioDyne, Estonia) following the manufacturer's instructions. A final volume of 20 $\mu$ l reaction mixture contained 4 $\mu$ l of the kit Master Mix, 0.5 $\mu$ l of forward and reverse primers (10 $\mu$ M), 2 $\mu$ l cDNA and 13 $\mu$ l nuclease-free water. The Quantitative real-time polymerase chain reaction was carried out in 7500 Fast real-time plates (Applied Biosystems) with the following cycling program: Initial activation at 95°C for 12 mins at one cycle followed by 40 cycles of denaturation 95°C for 15 s, annealing 62°C for 30 seconds, and Elongation 72°C for 30 seconds. Table 1 shows the primer sets used to amplify the genes. The quantification cycle, also known as the

threshold cycle (Ct), was calculated, and Relative mRNA expression levels of target genes were normalized to  $\beta$ -Actin using the 2- $\Delta\Delta$ Ct method [30]. The melting curve was evaluated to ensure PCR target specificity. A 4% agarose gel electrophoresis (Fig. 3c) was also used to determine the expected PCR product size was produced.

## 2.10 Evaluating the Toxicity of *Annona muricata* Ethanolic Fruit Extract in BALB/c Mice

Thirty male Balb/c mice aged 7- 8 weeks, weighing between 26- 30g, were bred in the animal house at JICA SAFARI House, Jomo Kenyatta University of Agriculture Technology Kenya. The BALB/c mice were acclimatized one week before the commencing of the study. The mice were then randomly divided into five groups of treatments with six mice per group. Group 1 was taken as controls and received normal saline only, while Groups 2, 3, 4, and 5 were given 3mg/kg, 10mg/kg, 30mg/kg, and 90mg/kg of *Annona muricata* ethanolic fruit extract, respectively *ad libitum* after sterilization by filtration. Dosage calculations were adapted from previously reported studies [32,33]. The care and handling of the animals were carried out according to OECD 2000 and 2008 guidelines.

## 2.11 Clinical Observations and Body Weight Measurements

The acute toxicity of *Annona muricata* fruit extract in mice was done for two weeks. The mice were kept under environmental conditions (23–25°C, 12 h/12 h light/dark cycle) and had access to commercial pelleted mice feed and water *ad libitum*. During 14 days, the animals were observed for clinical and physical activity abnormalities, toxicological symptoms. Body weights of live mice were measured on days 1, 7 and 14, to depict any toxic effect of the *Annona muricata* ethanolic fruit extract.

**Table 1. Primers used for Quantitative real-time polymerase chain reaction in this study**

Gene	Primers	Annealing Temp (°C)	PCR product size (bp)
<i><math>\beta</math>-Actin</i>	F 5'-CGGCATCGTCACCAACTG-3'	62	153
	R 5'-AACATGATCTGGGTCATCTTCTC-3'		
<i>c-Myc</i>	F 5'-CTGAGACAGATCAGCAACAACC-3'	62	129
	R 5'-TTGTGTGTTTCGCCTCTTGAC-3'		
<i>FGFR1</i>	F 5'-ATTTCTGTTGGCCT-3'	62	175
	R 5'-CTAGCGTCTTTGGGGAA-3'		

## 2.12 Termination of the Experiment

On the 15th day of the experiment, mice were starved for 4 hours and then subjected to carbon dioxide asphyxiation. Blood for hematology and biochemistry analysis was then collected through cardiac puncture. Biochemical analysis was done for the alanine aminotransferase, Aspartate aminotransferase, and Urea using standard diagnostic test kits on an automated clinical Biochemistry analyzer (Reflation Plus System®, MODEL: Cobas Analyzer India), and the biochemical results were compared with the normal ranges of BALB/c mice [34,35]. Samples for hematology were assessed for the following blood parameters; total and differential white blood cells (neutrophil and lymphocyte), total red blood cells (RBC), hemoglobin (Hb), MCV (mean corpuscular volume), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), Hematocrit and platelets were analyzed using automatic hematology analyzer (Mindy BC500).

## 2.13 Statistical Analysis

The data obtained from the current study were analyzed using Graph Pad Prism version 8.4.3. Software (Graph Pad Software, San Diego, CA, USA). One way ANOVA followed Dunnett's multiple comparisons test was applied to compare each group with the control. All the data were presented as mean  $\pm$  SEM (standard error of the mean). Statistical significance was considered at  $P \leq 0.05$  [28].

## 3. RESULTS

### 3.1 Determination of Cytotoxicity

Results obtained from the resazurin metabolic assay were used to determine the  $IC_{50}$  and  $CC_{50}$  values of *Annona muricata* ethanolic fruit extract and doxorubicin at all-time points (24hr, 48hr, and 72hr). Log dose-response curve (Fig. 1A) for *Annona muricata* ethanolic fruit extract was plotted for 24h, 48h, and 72h time points, and  $IC_{50}$  values were generated per time point are summarized in Table 2. The extract inhibited cell growth on RD cells in a time-dependent manner with the highest  $IC_{50}$  value of 18.43 $\mu$ g/ml after more prolonged exposure at 72hours.  $IC_{50}$  values obtained at time-points for *Annona muricata* ethanolic fruit extract were compared to  $IC_{50}$  values of doxorubicin (reference anticancer drug). Log dose-response curve (Fig. 2A) was used to analyze  $IC_{50}$  of doxorubicin at 24, 48,

and 72-time points and are summarized in Table 2. Doxorubicin had the highest cytotoxicity effects on RD cells at 72h with  $IC_{50}$  (3.14 $\mu$ g/ml) 5 times lower than the extract. However, the extract exhibited selective cytotoxicity in the control cells (Vero) with higher SI of 6.10 (Table 3) at 72hr values compared to doxorubicin (1.38). This implies that the more prolonged exposure of *Annona muricata* ethanolic fruit extract to the RD cells, the greater the inhibition of cells, while the more prolonged exposure to non-target cells (Vero) has negligible cytotoxic effects on the cells. Figs. 1A and 2B show the log dose-response curve of *Annona muricata* ethanolic fruit extract and doxorubicin, respectively, against Vero cells at time points.

Analyzed by one-way ANOVA followed by Tukey, Values for  $IC_{50}/CC_{50}$  are expressed as Mean  $\pm$  SEM, values with the different superscript letters in each row are statistically significantly different ( $p \geq 0.05$ ).

### 3.2 Selectivity Index of *Annona muricata* Ethanolic Fruit Extract and Doxorubicin

The selectivity index is derived from the  $IC_{50}$ , and  $CC_{50}$  values analyzed. Selectivity index values of 2 or greater than two are considered highly selective, while less than 2 indicates less selectivity [30]. From the results shown in Table 3, it is notable that the extract had higher selectivity index at all-time points compared to doxorubicin which was less selectivity to the normal cells at all-time points.

### 3.3 Effects of *Annona muricata* ethanolic Fruit Extract on mRNA Expression Levels of *c-Myc* and *FGFR1* Genes

The mRNA expression levels of *c-Myc* and *FGFR1* genes in RD cells was quantified using a Quantitative real-time polymerase chain reaction. The experiment was undertaken at three different time intervals of 24hrs, 48hrs and 72 hrs. The expression levels of the *c-Myc* and *FGFR1* genes were normalized using beta-actin gene (house-keeping gene). In RD cells treated with *Annona muricata* ethanolic fruit extract, the *c-Myc* gene was under expressed in a time-dependent manner, with the highest significant fold change of (3.4 folds) occurring at 72 hours compared to control. On the other hand, *c-Myc* was also under-expressed in RD cells treated with doxorubicin with (6.8

fold) at occurring at 72hrs compared to the extract and control. However, the differences in fold changes between doxorubicin and *Annona muricata* ethanolic fruit extract on *c-Myc* expression levels at 72 hours were statistically insignificant  $p \geq 0.05$  (Table 4). Similarly, the *FGFR1* gene was under-expressed in RD cells treated with *Annona muricata* ethanolic fruit extract compared to control and doxorubicin with the highest fold change (6.1) at 72hrs. However, it should also be noted that the differences in fold change between *Annona muricata* ethanolic fruit extract and doxorubicin on expression levels of *FGFR1* at 48hr and 72hr were not statistically significant  $p \geq 0.05$  (Table 4). Fig. (3A-B) show the effects of *Annona muricata* ethanolic fruit extract on mRNA expression levels of *c-Myc* and *FGFR1* genes in RD cells compared to the control (untreated cells) and doxorubicin in time points. Table 4 summarises the relative fold changes of the extract and doxorubicin. Fig. 3C shows

Agarose gel image of select qPCR amplicons of Beta-actin, *c-Myc*, and *FGFR1* genes of *Annona muricata* ethanolic fruit extract treated RD cells at different time intervals.

### 3.4 Clinical Observation and Body Weight

Oral administration of *Annona muricata* ethanolic fruit extract did not cause any signs or symptoms of toxicity in mice, including the highest dose tested. Signs of toxicity such as; changes in the skin, fur, watery eyes, sleep, salivation, diarrhea, and physical activity were not observed among treatment groups. An increment in body weight was observed from day-1 to day-14 across all dosage levels. Treatment group's weights were not substantially different from the controls ( $P \leq 0.05$ ). This result confirms the safety of *Annona muricata* ethanolic fruit extract tested on mice since no weight alteration was observed (Fig. 4).

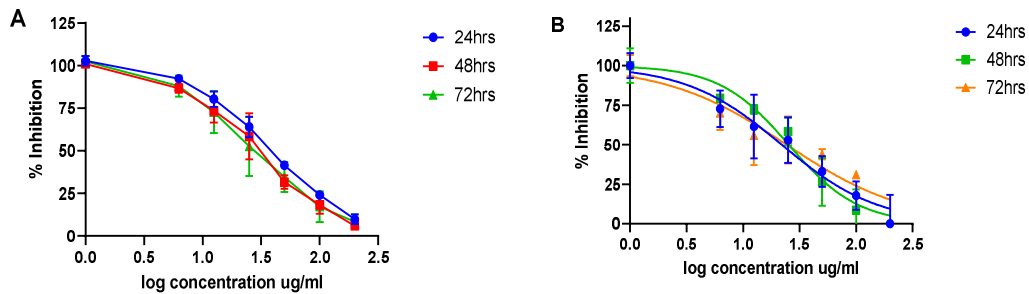


Fig. 1. Graphs showing log dose-response curves of % growth inhibition of RD cell (1A) and Vero cells (1B) treated with *Annona muricata* ethanolic fruit extract for 24, 48, and 72hrs, determined using the resazurin metabolic assay

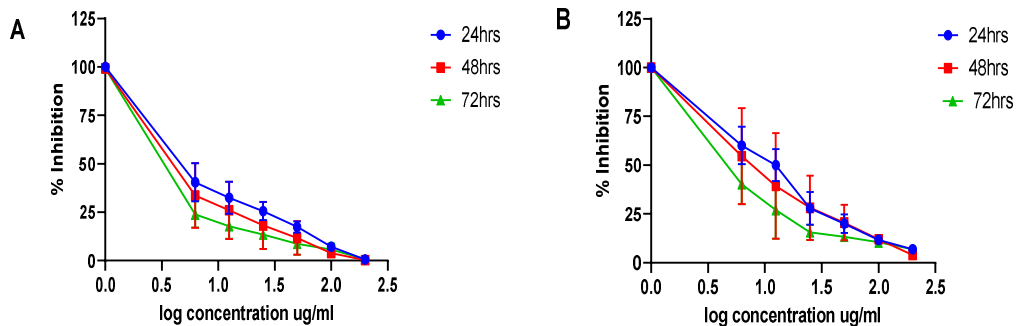


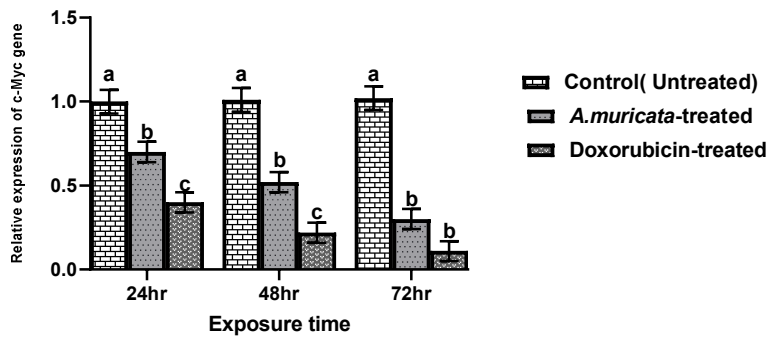
Fig. 2. Graphs showing the Log dose-response curves of % growth inhibition of RD cells (2A) and Vero cells (2B) treated with doxorubicin for 24, 48, and 72hrs, determined using the resazurin metabolic assay

**Table 2. Summary of IC<sub>50</sub> and CC<sub>50</sub> values of cell line with treatments**

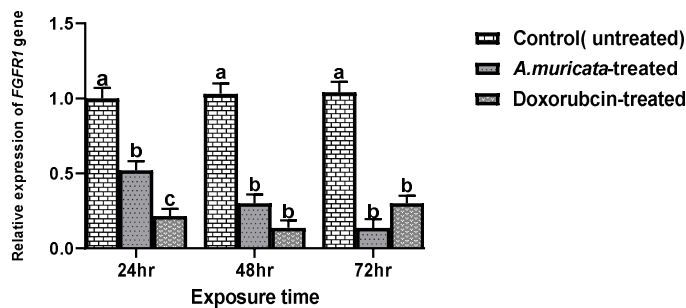
Treatment	Exposure time	IC <sub>50</sub> ug/ml ( RD cells)	CC <sub>50</sub> ug/ml ( Vero cells)
<i>Annona muricata</i> ethanolic fruit extract	24hrs	44.71± 0.02 <sup>a</sup>	133.6± 0.04 <sup>a</sup>
	48hrs	26.33 ± 0.04 <sup>b</sup>	120.5± 1.05 <sup>b</sup>
	72hrs	18.43± 0.52 <sup>c</sup>	112.60± 0.67 <sup>b</sup>
Doxorubicin	24hrs	9.61± 0.07 <sup>d</sup>	19.36±0.04 <sup>c</sup>
	48hrs	5.9 ± 0.15 <sup>e</sup>	9.10 ±0.02 <sup>d</sup>
	72hrs	3.41±0.04 <sup>f</sup>	4.71±1.23 <sup>e</sup>

**Table 3. Selectivity index of the treatments**

Cell line	<i>Annona muricata</i> ethanolic fruit extract			Doxorubicin		
	24 hrs	48hrs	72hrs	24 hrs	48 hrs	72hrs
Vero cells CC <sub>50</sub> (µg/ml)	133.6	120	112.60	19.32	9.10	4.71
RD cells IC <sub>50</sub> (µg/ml)	144.71	26.33	18.43	9.61	5.9	3.41
Selectivity index	2.98	4.5	6.10	2.0	1.54	1.38



**Fig. 3A.** the effect of *Annona muricata* ethanolic fruit extract on *c-Myc* mRNA expression was evaluated using the qPCR result.  $\beta$ -actin gene (house-keeping gene) was used to normalize the expression. The graph shows a time-dependent down-regulation of *c-Myc* after treatment with *Annona muricata* ethanolic fruit extract and doxorubicin. The different letter at each time of exposure depicts significant difference( $p \leq 0.05$ ).



**Fig. 3B.** Effect of *Annona muricata* ethanolic fruit extract on *FGFR1* mRNA expression was evaluated using qPCR analysis. *B-actin* (Housekeeping gene) was used for the normalization of the mRNA expression. The graph shows a time-dependent down-regulation of *FGFR1* after treatment with *Annona muricata* ethanolic fruit extract compared to doxorubicin (positive control for cancer drug). The different letter at each time of exposure depicts significant difference ( $p \leq 0.05$ )



Table 4. Summary of relative fold changes of the target gene

Gene	Time of exposure	<i>Annona muricata</i> ethanolic fruit extract		Doxorubicin	
		Fold change		Fold change	p-value
<i>c-Myc</i>	24hr	1.5		2.5	0.0097
	48hr	2.1		4.6	0.0095
	72hr	3.4		6.8	0.2435
<i>FGFR1</i>	24hr	1.9		3.1	0.0060
	48hr	3.6		4.3	0.7662
	72hr	6.1		3.3	0.3062

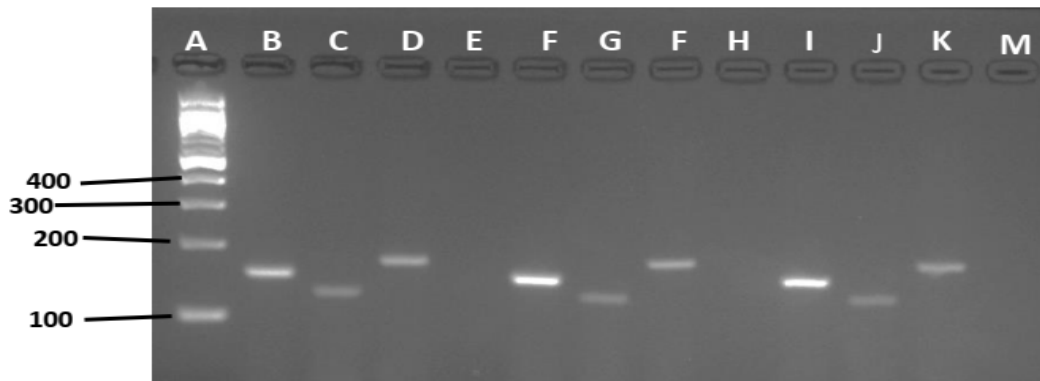


Fig. 3C. Agarose gel image of select qPCR amplicons of *Beta-actin*, *c-Myc*, and *FGFR1* genes of *Annona muricata* ethanolic fruit extract treated RD cells at different time intervals. Lane A represents ladder (100bp). Lanes E, H, and M, Negative Control. Lanes B, F, and I, *Beta-actin* gene (153bp) at 24hrs, 48hrs, and 72 hrs time intervals, respectively. Lanes C, G, and J *c-Myc* gene(129bp) at 24hrs,48hrs, and 72hrs, respectively, and Lane D, F, K-*FGFR1*(175bp) at 24hrs, 48hrs, and 72hrs, respectively

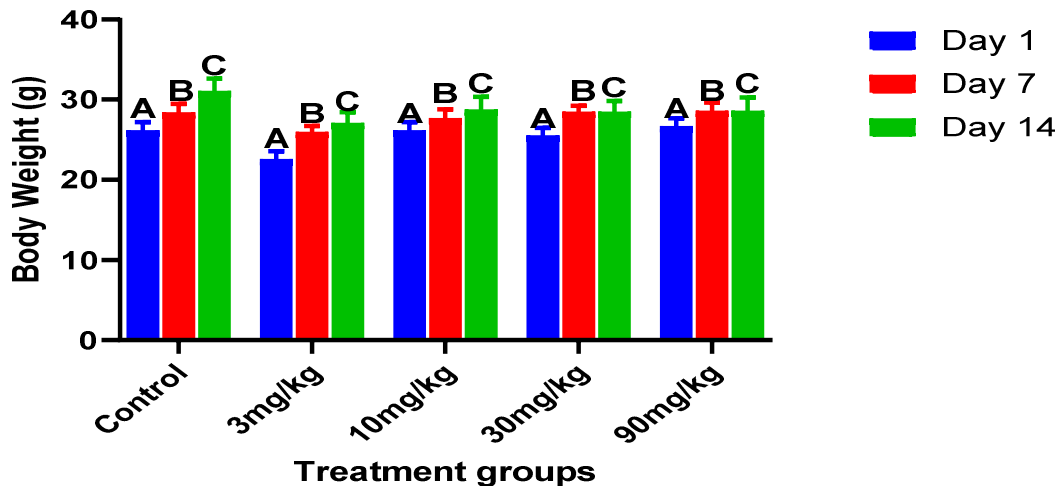


Fig. 4. Effects of *Annona muricata* ethanolic fruit extract on body weight of mice on different days. Values are expressed as mean  $\pm$  SEM (n = 5) and analyzed with one-way ANOVA followed by Dunnett's post hoc test. Mean values bearing the same letters among treatment groups in the same days show no significantly different ( $p \geq 0.05$ )

### 3.5 Effects of *Annona muricata* Ethanolic Fruit Extract on Liver and Kidney Biochemical Parameters

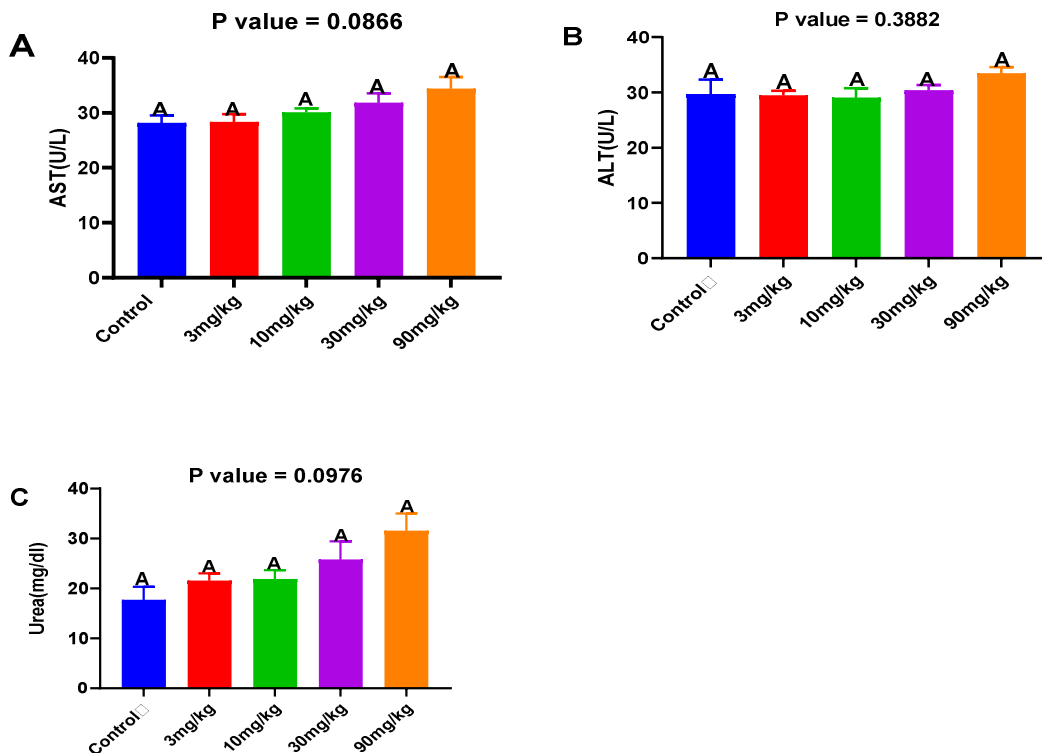
The effects of *Annona muricata* ethanolic fruit extract on liver and kidney biochemical parameters are summarized in Fig. 5. Similar to the kidney function parameter (urea –Fig. 5C), no significant differences were noticeable on liver function parameters; Alanine aminotransferase (ALT – Fig. 5B) and Aspartate aminotransferase (AST – Fig. 5A) between the treatment groups and control ( $p \geq 0.05$ ).

### 3.6 Effects of *Annona muricata* Ethanolic Fruit Extract on Hematological Parameters

The effects of *Annona muricata* ethanolic fruit extract on hematological parameters are

presented in Table 5. No significant difference was observed on hematological parameters; hemoglobin, total Red blood cells, Hematocrit, Mean Cell Hemoglobin, White blood cells, neutrophils, lymphocytes, and platelet count among the control group and treated groups (Table 5). However, the significant difference was only observed in MCHC among the groups ( $p > 0.03$ )

Values are expressed as Mean  $\pm$  SEM. Values that do not share a superscript letter are significantly different. ( $p \leq 0.05$ ); analyzed by ANOVA followed by Tukey's post hoc test. RBC- Red Blood Cells, Hb-Hemoglobin, HCT- Hematocrit, MCV-Mean Cell Volume, MCH-Mean Cell Hemoglobin, MCHC- Mean Cell Hemoglobin Content), WBC-White Blood Cells, PLT-Platelets, LYM- Lymphocytes and NEU- Neutrophils.



**Fig. 5. Effects of *Annona muricata* fruit extract on biochemical parameters: (A) – Mean Aspartate aminotransferase (AST) of mice; (B) – Mean alanine aminotransferase (ALT) of mice; (C) – Mean Urea of mice. Control is the group of mice without the treatment; Mean values bearing the same letter are not statistically significantly different from each other ( $p \geq 0.05$ ). SEM (n = 6)**

Table 5. Effects of *Annona muricata* ethanolic fruit extract on hematological parameters

Hematological parameters and indices	Treatments					P-value
	Control	3mg/kg	10mg/kg	30mg/kg	90mg/kg	
RBC(10 <sup>9</sup> /L)	10.06 ± 0.30 <sup>a</sup>	9.24 ± 0.07 <sup>a</sup>	10.02 ± 1.04 <sup>a</sup>	9.08 ± 0.09 <sup>a</sup>	10.28 ± 0.25 <sup>a</sup>	0.4005
Hb(g/dL)	15.16 ± 0.4 <sup>a</sup>	14.96 ± 0.31 <sup>a</sup>	15.67 ± 1.74 <sup>a</sup>	14.5 ± 0.45 <sup>a</sup>	15.6 ± 0.20 <sup>a</sup>	0.8723
HCT%	49.96 ± 1.73 <sup>a</sup>	46.56 ± 0.9 <sup>a</sup>	47.03 ± 0.9 <sup>a</sup>	44.26 ± 0.50 <sup>a</sup>	51.3 ± 0.9 <sup>a</sup>	0.4626
MCV(fL)	49.63 ± 1.04 <sup>a</sup>	49.63 ± 0.9 <sup>a</sup>	47.0 ± 0.36 <sup>a</sup>	48.73 ± 0.10 <sup>a</sup>	48.25 ± 1.35 <sup>a</sup>	0.9132
MCH(pg)	15.06 ± 0.03 <sup>a</sup>	15.9 ± 0.15 <sup>a</sup>	15.6 ± 0.15 <sup>a</sup>	15.96 ± 0.6 <sup>a</sup>	15.25 ± 0.55 <sup>a</sup>	0.1218
MCHC (g/dL)	30.36 ± 0.58 <sup>bb</sup>	32.0 ± 0.43 <sup>ab</sup>	33.2 ± 0.55 <sup>aa</sup>	32.73 ± 1.35 <sup>ab</sup>	31.5 ± 0.2 <sup>ab</sup>	0.0367
WBC (x10 <sup>9</sup> /L)	7.70 ± 0.78 <sup>a</sup>	7.06 ± 0.8 <sup>a</sup>	7.46 ± 0.94 <sup>a</sup>	9.46 ± 1.36 <sup>a</sup>	9.01 ± 1.36 <sup>a</sup>	0.6546
NEU (x10 <sup>9</sup> /L)	1.76 ± 0.15 <sup>a</sup>	1.116 ± 0.48 <sup>a</sup>	0.74 ± 0.33 <sup>a</sup>	1.29 ± 0.59 <sup>a</sup>	1.02 ± 0.35 <sup>a</sup>	0.2961
LYM (x10 <sup>9</sup> /L)	5.93 ± 0.71 <sup>a</sup>	5.73 ± 1.1 <sup>a</sup>	6.71 ± 0.90 <sup>a</sup>	8.15 ± 1.93 <sup>a</sup>	8.3 ± 1.13 <sup>a</sup>	0.5986
PLT(x10 <sup>9</sup> /L)	1074 ± 308.4 <sup>a</sup>	848.66 ± 136 <sup>a</sup>	803.6 ± 113 <sup>a</sup>	909.3 ± 107 <sup>a</sup>	1009.5 ± 3.5 <sup>a</sup>	0.8642

#### 4. DISCUSSION

Cell proliferation is the process that increases the number of cells and is defined by the balance between cell division and cell loss through cell death or differentiation [36]. This process is increased in cancerous cells, leading to tumor aggressiveness [37]. Drugs or natural products that inhibit the spread of cells into surrounding tissue, are known to have antiproliferative activity [38]. The present study evaluated the antiproliferative activity of *Annona muricata* ethanolic fruit extract in the human rhabdomyosarcoma cell line (RD) and Vero cell lines. The antiproliferative activities of the extracts were reported based on median inhibitory concentration ( $IC_{50}$ ) [38]. This study showed that the *Annona muricata* ethanolic fruit extract exhibited cytotoxic effects towards the RD cells in a time- and dose-dependent manner with highest  $IC_{50}$  of 18.17  $\mu$ g/ml at occurring at 72hr. These findings are in agreement with other previous findings where aqueous acetone extract and Methanol and dichloromethane (DCM) of fruit extract significantly inhibited cell growth of Human BCMDA-MB-468, MCF-7 [39], DU145 (prostate cancer, HTB-81), HCC 1395 (breast cancer, CRL-2324), Hela (cervical cancer cell lines, CCL-2) [21,26]. Doxorubicin, the positive control, had lower  $IC_{50}$  values at all-time points than those of *Annona muricata* ethanolic fruit extract, this result is justifiable because doxorubicin is a purified compound as opposed to the *Annona muricata* ethanolic fruit extract, which is in crude form. Selective cytotoxicity is an important mechanism that justifies an ideal chemotherapeutic agent [18]. A drug with an SI of 2 or greater than two is considered highly selective [30]. The *Annona muricata* ethanolic fruit extract had a higher selectivity index on Vero cells compared to the reference cancer drug doxorubicin. These results suggest that the extracts can discriminate between malignant and non-malignant cells hence considered safer when compared to doxorubicin. These findings are consistent with the previous studies that showed that *Annona muricata* contain bioactive compounds that more toxic to cancer cells than normal cells [23,24,26].

In tumorigenesis, metastasis, cell proliferation, differentiation, anti-apoptosis, and angiogenesis are all critical hallmarks of cancer [12]. Deregulation of the *c-Myc* and *FGFR1* genes in rhabdomyosarcoma causes uncontrolled cell proliferation, immune evasion, cell immortalization, metastasis, genomic instability,

and uncontrolled angiogenesis [14,40,41]. As a result, rhabdomyosarcoma patients experience tumor aggressiveness, medication resistance, and a catastrophic prognosis. [42,43]. In cancer research, several lines of evidence have shown that bioactive compounds found in natural products tend to influence gene expression by regulating critical transcription factors involved in tumorigenesis [44]. The current study determined the effects of *Annona muricata* ethanolic fruit extract on the expression of *c-Myc* and *FGFR1* genes in RD cells. The expression levels were assessed using real-time qPCR at three different times: 24 hours, 48 hours, and 72 hours. Results obtained in this study revealed that the *c-Myc* gene expression was downregulated by *Annona muricata* ethanolic fruit extract in a time-dependent manner compared to control (Figure 3A), with the highest fold change (3.4) registered at 72 hours. Comparatively, no significant changes in *c-Myc* gene expression levels were observed with the doxorubicin ( $p \geq 0.02435$ ). Essential signaling pathways, such as the Wnt/beta-catenin, Ras/Raf/ERK, and the Ras/PI3K/AKT/GSK-3 pathways, regulate the expression of intracellular *c-Myc* [15]. The expression of intracellular *c-Myc* is regulated by signaling pathways such as Wnt/beta-catenin, Ras/Raf/ERK, and Ras/PI3K/AKT/GSK-3. In addition, the control of *c-Myc* gene expression has a cascade effect on genes involved in cell cycle progression, proliferation (MINA53 and PTMA), and metabolism (CAD, LDHA, and ODC1) [41]. Our findings plausibly suggest that *Annona muricata* ethanolic fruit extract inhibits RD cell growth in the antiproliferative assay by altering critical signaling pathways which modulate *c-Myc* gene expression. These findings align with previous research that showed plant extracts from Curcumin, *Punica granatum*, and *S. herbacea* contain therapeutic properties that down-regulate the *c-Myc* gene expression in different adenocarcinomas [41,45,46].

Similarly, the *FGFR1* gene was also downregulated in a time-dependent manner in RD cells, treated with *Annona muricata* ethanolic fruit extract compared to control with the highest relative fold change (6.1) occurring at 72hr. However, compared to the standard drug, the *FGFR1* was upregulated at 72hrs, but the difference was not statistically significant  $p \geq 0.05$ . Angiogenesis is a complex process associated with a series of events such as endothelial, cell migration, and invasion. These complex events rely on *FGFR1* signaling and *FGFR1* phosphorylation to initiate downstream signaling

pathways [47]. Our findings have demonstrated that *Annona muricata* ethanolic fruit extract could have a potential effect on the *FGFR1*-mediated PI3K-Akt signaling pathway involved in the phosphorylation of *FGFR1*, leading to the suppression of RD cell growth and angiogenesis. Hence *Annona muricata* ethanolic fruit extract could be a potential new therapeutic agent due to its anti-tumor and anti-angiogenesis mechanisms against rhabdomyosarcoma. The present findings agreed with the earlier studies, which reported natural products inhibiting the *FGFR1* pathway, leading to anti-angiogenesis in the cancer cells [46–48]. These data further suggest that the *Annona muricata* ethanolic fruit extract could be an effective chemotherapeutic treatment for rhabdomyosarcoma as it has shown that it has therapeutic effects of suppressing the expression levels *c-Myc* and *FGFR1*.

For centuries, medical plants have been considered efficacious and safe due to reducing severe side effects [49], consequently leading to prolonged usage of herbal medicine and formulation by individuals without proper dosages and scientific knowledge of their toxicological effects [50]. Therefore, scientific knowledge towards Oral toxicity data is required to predict the safety associated with medical products and helps identify dosages that could consider safe for humankind [51] during the *Annona muricata* ethanolic fruit extract oral toxic in the current study. No symptoms or signs of clinical and physical activity changes were observed compared to treated mice and control. Additionally, all animals gained weight across all dosage levels, and the weights of the treated groups were not significantly different compared to controls ( $P \leq 0.05$ ). These results confirm the extract's safety on mice treated up to 90mg/kg since no signs of adverse toxicity and body weight alteration were observed. This finding agrees with a study conducted by [52] whereby lyophilized fruit juice of *Annona muricata* showed no bodyweight alteration among treated and control rodents.

Alanine aminotransferase and aspartate aminotransferase are enzymes in the liver which determine liver toxicity caused by drugs, chemicals, and formulations [49]. In the current work, *Annona muricata* ethanolic fruit extract showed no effect on ALT and AST levels of treated mice compared to the control group ( $p > 0.05$ ). Correspondingly, the kidney function was also evaluated for potential toxicological effects

induced by the extract through measurement of urea concentration. Any significant change in this parameter could point to induced nephrotoxicity. However, the obtained data indicated no adverse effect on urea in the treated mice when compared to the control group ( $p > 0.05$ ). Our findings echo those of a previous study conducted elsewhere [20], suggesting that *Annona muricata* fruit is safe and not toxic and could as well be used for therapeutic purposes.

The hematopoietic system is an essential target for toxic compounds and evaluating animals and humans physiological and pathological states [51]. Red blood cell indices such as the mean corpuscular volume (MCV), the mean corpuscular hemoglobin concentration (MCHC), and Hematocrit, hemoglobin (HB) are vital indicators for the diagnosis of different types of anemia [53]. In the current study, the effect of *Annona muricata* ethanolic fruit extract on MCV, MCH, and HB, and HCT were not statistically significantly different compared to the control group. These findings demonstrate that the *Annona muricata* ethanolic fruit extract at both doses did not cause a significant toxic effect on the levels of calculated red blood cell (RBC) indices. Furthermore, the *Annona muricata* ethanolic fruit extract did not cause any macrocytic, hemolytic, hypochromic, and microcytic anemias, as the size and concentration of hemoglobin in RBC were not affected in treated mice. These findings agree with other findings that showed values of RBC parameters of extract-treated Wistar rats did not show any anemia [54].

White blood cells are defense mechanisms that respond to infectious agents, tissue injury, or any inflammation in the body [50]. In the hematological analysis done in this study, the effect of the fruit extract was also evaluated on the white blood cell (WBC) count. Like previous studies [55], the WBC count in this study demonstrated no significant difference compared to the control. This result may signify that that *Annona muricata* ethanolic fruit extract does not possess toxins suppressing the average production of WBC (leucopenia) or inducing leukocytosis.

Platelets have a significant role in blood-clot formation during tissue damage hence preventing blood loss. Thrombocytopenia is associated with a reduced number of platelets caused by decreased production or increased destruction of platelets. Thrombocytopenia is

usually non-immune (drug-induced) or Immune thrombocytopenia caused by an antibody. On the other hand, thrombocytosis is an abnormal increase in circulating platelets due to toxic agent-mediated inflammation or abnormal bleeding [34]. There was no significant difference in the platelet count between treatment groups compared to the control group in this study. These results suggest that the *Annona muricata* ethanolic fruit extract does not affect inducing thrombocytopenia or thrombocytosis. Based on these findings of the acute toxicity *Annona muricata* ethanolic fruit extract may be safe and non-toxic for pharmacological and therapeutic reasons. Additionally, the results of this acute toxicity investigation aided in the selection of acceptable extract test doses for further *in vivo* anticancer activity testing.

## 5. CONCLUSION

This study has shown that the *Annona muricata* ethanolic fruit extract exhibits anticancer effects by inhibiting Human rhabdomyosarcoma cells through downregulation of vital molecular markers (*FGFR1* and *c-MYC* genes) involved in tumorigenesis of rhabdomyosarcoma. Furthermore, the extracts showed no toxicity effect in all doses studied and were relatively selective, as shown by the high selectivity index. These findings suggest that the *Annona muricata* ethanolic fruit extract could be a potential chemotherapeutic agent in the treatment of rhabdomyosarcoma. Nevertheless, further investigations should be done *in vivo* further to determine its anti-tumor activity and subacute and chronic toxicity further to explore the safety profile of this medicinal plant and undertake studies in non-human primates, a step towards clinical utilization of the plant.

## SUPPLEMENTARY MATERIALS

Supplementary material is available in the following link:

<https://www.journaljocamr.com/index.php/JOCAMR/libraryFiles/downloadPublic/6>

## AVAILABILITY OF DATA AND MATERIALS

Raw data can be obtained from the corresponding authors upon reasonable request.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

The experimental protocol adopted in this study was approved by JKUAT Institution Ethics Review Committee (JKU/IERC/02316/0028).

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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