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INTEGRATIVE INVESTIGATION OF *MANILKARA KAUKI* PHYTOEXTRACT: ELUCIDATING MULTIFACETED ANTINEOPLASTIC MECHANISMS FROM CELLULAR VIABILITY MODULATION TO *IN VIVO* TUMOR REGRESSION

Medha Mohan Hegde[1,3], K. Lakshman[1], Mukund Handral[2]*

Address(es):
[1] PES University, Faculty of Pharmaceutical Sciences, Department of Pharmacognosy, Bangalore,560100, Karnataka, India.
[2] PES University, Faculty of Pharmaceutical Sciences, Department of Pharmacology, Bangalore,560100, Karnataka, India.
[3] Rajiv Gandhi University of Health Sciences, Nargund College of Pharmacy, Department of Pharmacognosy, Bangalore, 560085, Karnataka, India.

*Corresponding author: medhaks@gmail.com

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ABSTRACT

Cancer, a complex and profound ailment, needs new treatment ideas to address its pervasive worldwide implications. Within this context, *Manilkara kauki*, a relatively unexplored tropical botanical plant, emerges as a reservoir of significant potential due to its inherent bioactive properties. Its traditional uses and preliminary investigations suggest it might open up a new path in cancer treatment. The present study investigated the promising antitumor activity of the ethanolic extract of *Manilkara kauki* (EEMK) by undertaking an integrated investigation via *in vitro* and *in vivo* assessments. The cytotoxic effect of EEMK was meticulously evaluated by 2 discrete assays, namely Trypan Blue and MTT at doses of 50,100,200,400 and 800 µg/ml, and apoptosis induction on EAC cells was studied by DAPI staining at dose IC50 value ~265 µg/ml. Further antitumor activity was tested at 200 and 400 mg/kg in Erlich ascites carcinoma-bearing mice. The outcomes from both cytotoxic assays consistently revealed a concentration-dependent decline in cell viability, as discerned from IC50 values ~265 µg/ml and ~276 µg/ml respectively. DAPI staining confirmed the apoptosis induction in EAC cells. Transitioning to an *in vivo* milieu, EEMK demonstrated pronounced antitumor efficacy in mice bearing experimentally induced tumors by substantial reductions in tumor volume, and tumor weight, alongside a commendable extension in mean survival time and bringing back the hematological parameters to normal. Furthermore, EEMK exhibited hepatoprotective attributes, effectively mitigating SGPT and SGOT levels. These findings serve as a pivotal cornerstone in unraveling the multifaceted mechanisms underlying EEMK's interventions within cancer management.

Keywords: Cytotoxicity, Antitumour, *Manilkara kauki*, EAC

INTRODUCTION

Cancer is a broad and complex group of diseases characterized by the unchecked growth and spread of abnormal cells throughout the body. Its global prevalence poses a significant public health challenge, witnessing approximately 19.3 million new cancer incidences and 10 million cancer-related mortalities in the year 2020 alone **(Sung *et al*., 2021)**. As a primary cause of morbidity and mortality on a worldwide scale, cancer imposes substantial physiological, psychological, and economic hardships upon individuals, families, and entire societies **(Valdez & Brennan, 2018; Antoni *et al*., 2023)**. Managing this intricate array of diseases necessitates a comprehensive and interdisciplinary approach to advance our understanding and develop effective interventions **(Yang *et al*., 2019; Higdon *et al*., 2018)**. Cancer's impact exhibits considerable variation among diverse regions and populations, with certain countries and demographic groups experiencing disproportional effects **(Arnold *et al*., 2020; Mattiuzzi & Lippi, 2019; Cao *et al*., 2021).** As per the World Health Organization (WHO), the worldwide cancer burden is projected to escalate, reaching 27.5 million novel cancer incidents and 16.3 million cancer-related fatalities by 2040 **(*Global Health and Cancer Epidemiology | EGRP/DCCPS/NCI/NIH*).** The prevailing global cancer types include lung, breast, colorectal, prostate, and stomach cancer **(B. Wang *et al*., 2022; Nieto-Jiménez *et al*., 2022)**. A comprehensive understanding of these patterns aids in developing targeted strategies to address the specific challenges posed by various cancer types and their distributions, thus enabling more effective and personalized interventions to alleviate the burden of this complex disease **(Zhang et al., 2020; Navya *et al*., 2019; X. Wang *et al*., 2019)**.

Conventional cancer treatment procedures include surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy. Although these methods are effective in slowing down the growth of tumors and increasing the chances of survival, they also come with significant side effects, including fatigue, baldness, vomiting, and increased vulnerability to infections. Additionally, some cancers show resistance to standard therapies, which makes it necessary to investigate alternate therapeutic options that may provide better disease control and lessen side effects from treatment **(Pucci *et al*., 2019; Miller *et al*., 2019)**. In the field of oncology, finding novel and tailored treatments is crucial to addressing the different problems posed by different types of cancer and improving patient outcomes **(M. Wang *et al*., 2021; Meng *et al*., 2019)**.

For centuries, plants have served as vital reservoirs of medicinal substances, offering a plethora of alternative therapies for diverse ailments, including cancer. Numerous plants harbour bioactive compounds exhibiting notable anticancer attributes, manifesting as antioxidant, anti-inflammatory, and cytotoxic effects **(Feria & Times, 2024; Perera, 2019; Gezici & Şekeroğlu, 2019; Majolo *et al*., 2019)**. Notably, the tropical plant *Manilkara kauki* (Common name: Caqui), belonging to the Sapotaceae family within the genus Manilkara, emerges as a significant candidate in this realm of medicinal exploration. Its phytochemical constituents may hold promise in the development of novel therapeutic approaches for combating cancer and permit further investigation to disclose its full potential in oncological interventions. *Manilkara kauki* has been investigated for its potent antioxidant, antityrosinase, and anti-diabetic actions. This tropical plant boasts a diverse array of phytocompounds, including phenolic compounds, flavonoids, triterpenoids, and saponins, which have been investigated for their potential therapeutic applications **(T. K., 2012; Purba *et al*., 2023)**. In traditional Indian medicine, the bark and roots of *Manilkara kauki* have been traditionally employed as an astringent agent and a remedy for infantile diarrhea, seeds febrifuge, anthelmintic, antileprotic, and leaf used as a poultice for tumors **(Kirtikar, 2006; Khare, 2007)**. However, there isn't any scientific evidence for its antitumor activity. Hence, *in- vivo* Ehrlich Ascites Carcinoma model has been selected for evaluating the traditionally claimed antitumor activity of EEMK extract. It is a well-established model and has largely been used for the study of tumor pathogenesis and evaluation of anti-tumorigenic agents **(Ozaslan *et al*., 2011)**. Before the in-vivo animal model's design, in-vitro cytotoxicity tests were performed on EAC cells to gain a better understanding of the EEMK extract's possible anticancer properties.

The medicinal properties of *Manilkara kauki* remain relatively unexplored, but preliminary studies suggest potential therapeutic applications, particularly as an anticancer agent. This research paper aims to contribute to the growing body of knowledge on *Manilkara kauki's* cytotoxicity and antitumor properties, paving the

way for further exploration of this plant as an alternative therapy in oncological interventions. Additional research is required to comprehensively understand its potential health benefits and associated risks when used medicinally.

MATERIALS AND METHODS

Drugs and chemicals

The following media and chemicals were obtained from Hi-Media Laboratories, Mumbai: 5 FU (5 Fluorouracil), MTT (3 (4, 5 dimethylthiazol 2 yl) 2, 5 diphenyltetrazolium bromide), Trypan blue, FBS (Foetal bovine serum), Minimum essential media (#AL075A), Streptomycin sulfate, Penicillin G sodium salt, Trypsin-EDTA solution, DAPI dihydrochloride, Paraformaldehyde solution, Triton X-100, DMSO (Dimethyl sulfoxide), DPBS (Dulbecco's Phosphate Buffered Saline).

Collection of plant material

The leaves of *Manilkara kauki* were collected from Neyyattinkara, Trivandrum District, Kerala, India and identified and authenticated by Dr. S. Mutheeswaran (Ph.D.), a Scientist affiliated with Xavier Research Foundation at St. Xavier's College, Palayamkottai, Tamil Nadu, India. To ensure proper documentation and future reference, a voucher specimen has been deposited at the Department of Pharmacognosy, PES College of Pharmacy, Bangalore.

Preparation of extract

The leaves were subjected to shade drying and subsequently coarsely powdered. Soxhlet extraction was performed using 95% v/v ethanol as the solvent. The extraction yield was noted to be 24%. The obtained extract was made free from ethanol by concentrating under reduced pressure using a vacuum evaporator. To determine the phytochemical composition, the ethanol extract was subjected to preliminary phytochemical investigation, to check the presence of primary and secondary metabolites such as Alkaloids, Glycosides, Saponins, Phytosterols, Phenolics and tannins, Proteins, Fixed oils and fats, Carbohydrates, Gums and mucilage according to standard procedure (**Kokate, 2020**).

Animals

The Swiss albino mice, with an average weight of 25 ± 4 g were ethically acquired from Vertebrates which is a registered facility (Reg no-2138/po/RcBiBt/s/21/CPCSEA) situated on Magadi Road, Bangalore -02. Adequate care was taken to ensure the animals' well-being throughout the experiment. They were housed in spacious polypropylene cages within a controlled-temperature environment and had unrestricted access to standardized pelleted feed and clean drinking water. The experimental procedures strictly adhered to the guidelines set forth by the Committee for Control and Supervision of Experiments on Animals (CPCSEA) and were conducted with approval from the institutional animal ethical committee (PESCP/IAEC/139/2022). Ethical considerations were given utmost importance to ensure the welfare of the animals and compliance with established scientific standards.

In-vitro cytotoxicity studies

Tumor cells

Ehrlich's ascites carcinoma (EAC) cells were procured from the National Centre for Cell Sciences, Pune, India. cells were subcultured at 37°C in 5% CO_2 using a carbon dioxide incubator (Memmert, Germany), using minimal essential medium supplemented with 15% penicillin/streptomycin and Foetal bovine serum which was used for the *In-vitro* study. Later cells were propagated through intraperitoneal inoculation of 2×10^6 cells/mouse. It was used for in-vivo study.

Cell viability determination by Trypan Blue Assay

Cell viability assessment was conducted using the Trypan blue method, a widely accepted assay based on the principle of differential dye exclusion by live and dead cells. Added 200µl of EAC cell suspension containing 20,000 cells in triplicate onto a 96-well plate. After 24 hrs incubation period, test agents prepared in 0.1% DMSO, were added to their respective wells at (50, 100, 200, 400, and 800 µg/ml) concentrations. Incubated at 37°C with 5% CO_2 for 48 hours. After the incubation period, cells were trypsinized using 50µl of 0.25% trypsin-EDTA solution Mixture of 10µl of the cell suspension and 10µl of 0.4% trypan blue stain was taken for determination of cell viability using the LUNA -II automated cell counter (#L40002, South Korea). During the evaluation, viable cells, characterized by their intact membrane, remained unstained, while non-viable cells, with compromised membrane integrity, retained the blue dye **(Strober, 2015)**.
The percentage of cell viability was calculated using the following formula: [(number of viable cells / total number of viable and non-viable cells) x 100].

MTT Assay

The cytotoxicity of EEMK was assessed through the MTT assay, a colorimetric method for measuring cell proliferation and viability. Mitochondrial lactate dehydrogenase in live cells reduced the water-soluble tetrazolium dye MTT to formazan crystals, generating a purple color. The intensity of the color was directly proportional to the number of viable cells, measured spectrophotometrically at 570nm **(Kumar *et al*., 2018)**.
The MTT assay's reliability and accuracy were ensured through the incorporation of various controls. These included the medium control, which accounted for background absorbance using medium without cells, and the negative control (medium with cells but without the experimental drug/compound), where cells were exposed to medium alone to assess inherent cytotoxicity. MTT assay was carried out by 200µl of cell suspension containing 10,000 cells seeded in triplicate in a 96-well microtiter plate. The cells were allowed to grow overnight without the test agent. Subsequently, the test agents prepared using 0.1% DMSO at various concentrations (50, 100, 200, 400, and 800 µg/ml) were introduced into the wells. The plate was then incubated at 37°C with 5% CO_2 for 48 hours to allow the test agents to exert their cytotoxic effects. Following the incubation, the spent media was removed, and MTT reagent was added to each well at a final concentration of 0.5mg/ml. The plate was incubated for an additional 3 hours. Afterward, 100µl of solubilization solution (DMSO) was added to dissolve the formazan crystals, and the resulting solution's absorbance was read at 570nm using a spectrophotometer or ELISA reader (ELX-800, BioTek, USA).
The assay's principle relied on the reduction of MTT to formazan only by metabolically active and viable cells. Therefore, the amount of formazan dye directly reflected the number of viable cells **(Kumar *et al*., 2018)**.

Apoptosis induction assay

DAPI (4,6-diamidino-2-phenylindole dihydrochloride) staining was conducted to assess the nuclear morphology of cancer cell lines post-treatment with the ethanolic extract of *Manilkara kauki* (EEMK). 200µl of Ehrlich Ascites Carcinoma (EAC) cell suspension was seeded in a 96-well plate at a cell density of 10,000 cells per well. The cells were allowed to grow overnight without exposure to the test agent. Subsequently, cells were treated with IC50 value ~265 µg/ml from MTT assay. A controlled incubation period of 48 hours was maintained at 37°C in a 5% CO_2 atmosphere to facilitate cellular responses to the test agents. After incubation, the spent media was removed from the wells, and the cells were washed twice with phosphate-buffered saline (PBS). The cells were then fixed with 2% paraformaldehyde for 30 minutes at room temperature. Following fixation, the cells were washed with PBS and subsequently permeabilized with 0.1% Triton X-100 for 10 minutes **(Wallberg *et al*., 2016)**. To visualize the nuclei, the cells were incubated with DAPI (1µg/ml) for 5 minutes. Subsequently, the cells were examined under an Inverted fluorescence microscope (Zeiss Axio Observer 7, Germany).

Acute Oral Toxicity

The acute toxicity study of the EEMK was conducted in Swiss albino mice to assess its safety profile. At a dose level of 2000 mg/kg, no gross behavioral changes or mortality were observed, suggesting a high level of safety. The LD50 value of EEMK was estimated to be more than 2000 mg/kg, signifying its low toxicity. Subsequently, doses of 200 and 400 mg/kg of EEMK were selected for the present study based on the principle of effective dose calculation, considering 1/5 to $1/10^{th}$ of the lethal dose **(OECD, 2022)**.

In-vivo Anti-tumor activity

EAC carcinoma model

The animals were categorized into 5 groups (n =12), except group I which served as the normal control, all other groups received 2×10^6 EAC cells per mouse. II group served as disease control. After 24 hours of tumor inoculation, the III and IV groups received the EEMK extracts 200 and 400 mg/kg respectively and the V group received standard 5-Fluorouracil (20mg/kg) once daily for 9 consecutive days. Following 24 hrs from the last day, the last dose, and after 18 hours of fasting, blood was drawn from six animals from each group from direct cardiac puncture used for estimation of hematological and serum biochemical parameters. Later mice were sacrificed by cervical dislocation for collection of ascitic fluid to find out the tumor volume (TV), packed cell volume (PCV), and tumor weight. The other six animals from each group were maintained with food and water ad libitum to determine the mean survival time (MST) and percentage increase in life span. The antitumor activity of EEMK was determined in EAC-bearing animals by following observation **(Dolai *et al*., 2012)**.

Hematological parameters

Measurement of Haemoglobin (Hb)

The hemoglobin (Hb) content was measured using Sahli's hemoglobinometer (Acid haematin method). Twenty microliters of EDTA anti-coagulated blood were placed in a hemoglobinometer tube containing N/10 HCl up to 2g% and stirred. Then added drop by drop distilled water and stirred until the color matches with comparator. The final reading was noted from the tube reading.

Total WBC count

Dilute EDTA anticoagulant blood with WBC diluting fluid 1:20 in a WBC pipette, mixed properly. This mixture was observed using a hemocytometer (Neubauer's counting chamber), counted the WBC using a low power objective.
WBC count = (number of WBC counted x dilution factor)/(area x dept of the fluid)

Total count of RBC

Exactly 20 µl of EDTA anticoagulated blood to the tube containing RBC diluting fluid and mixed well. RBCs were counted using Neubauer's chamber under lower magnification.
RBC count per mm^3= N X 10000 (N= Total RBCs in 5 square)

Tumor growth response

The antitumor effect of EEMK was evaluated by tumor volume, tumor weight, Packed cell volume (PCV), viable and non-viable cell count, Mean survival time (MST), and % increase in lifetime (%ILS)

Tumor Volume, (PCV) Packed cell volume and Tumor Weight

The ascitic fluid was collected from the peritoneal cavity. The volume was measured using a graduated centrifuge tube, and packed cell volume was determined by centrifugation using a centrifuge (R-8C, Remi, India) at 1000 rpm for 5 min. Tumor weight was measured by taking the weight of the mice before and after the collection of the ascitic fluid from the peritoneal cavity.

Viable and Nonviable Tumor Cell Count

The ascitic fluid was diluted 20 times with PBS in a WBC pipette Then a drop of the diluted suspension was taken on the Neubauer chamber The cells were stained with 0.4% trypan blue dye. The cells that did not take up the dye were viable and those that took the stain were non-viable. Total number of viable and nonviable cells were counted by using the following formula:
Cell count = (number of cells x dilution factor)/(area x thickness of liquid film)

Percentage Increased Life Span (%ILS)

The effect of EEMK on tumor growth was monitored by recording the mortality of the experimental mice. The %ILS was calculated by the following formula:
%ILS = [(mean survival time of treated group/mean survival time of control group) – 1] × 100
where Mean Survival Time (MST) = (day of first death + day of last death)/2.

Biochemical parameters

The blood samples were allowed to clot at 4^0C for 45 min and the serum was separated by centrifugation at 5000 rpm for 10 min. Later it was used to estimate the (SGPT) serum glutamate pyruvic transaminase, (SGOT) Serum glutamic oxaloacetate transaminase. These parameters were analyzed by commercially available kits manufactured by Transasia Bio-medicals Ltd, Himachal Pradesh, India using semi-auto bioanalyzer, in Remi, India.

Statistical analysis

The results were expressed in Mean±SEM. The data obtained from the study were subjected to one-way ANOVA followed by Dunnett's Test using GraphPad Prism 10 software. P values less than 0.05 ($p< 0.05$) were considered as significant.

RESULTS

Phytochemical investigation of ethanol extract of *Manilkara kauki* showed the presence of secondary metabolites such as alkaloids, glycosides, triterpenoids, Tannins, saponins, and primary metabolites like carbohydrates.

In-vitro cytotoxicity studies

Trypan blue assay

Following a 48-hour incubation with varying concentrations of EEMK, the cell viability was determined. In the present study, EEMK exhibited concentration-dependent cytotoxic effects, hindering cell growth. Figure 1 depicts the cytotoxic effects of EEMK on EAC, with an increase in EEMK concentration, the cell viability decreased significantly to 28.04% at the highest concentration 800 µg/ml. The IC50 value for EEMK was approximately 276 µg/ml, suggesting its potential as an effective anti-cancer agent against EA carcinoma cells, supported by the concentration-dependent trend observed in the graph.

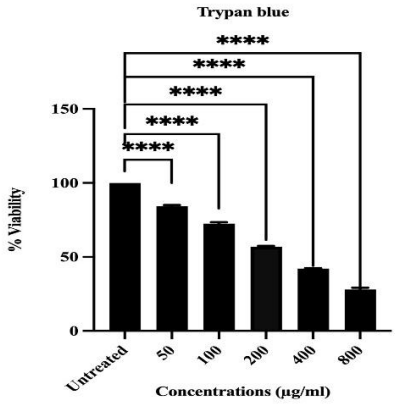

Figure 1 Dosage-dependent effect of EEMK on EAC cells in Trypan Blue assay, Values are represented as mean ± SEM (n = 3).

MTT Assay

In the present study, the cytotoxic effects of EEMK were evaluated on the EAC cells. The cells were treated with various concentrations ranging from 50-800 µg/ml after 48 hours of incubation. It is evident from Figure 2, that as the concentration of EEMK was increased, the viability decreased i.e., 50 µg/ml exhibited 82.99% viability which gradually decreased to 27.15% at 800 µg/ml. This demonstrated a concentration-dependent cytotoxicity with an IC50 value of ~265 µg/ml for EEMK. Notably, at 200 µg/ml and above, EEMK significantly reduced the viability suggesting its potentially higher efficacy.

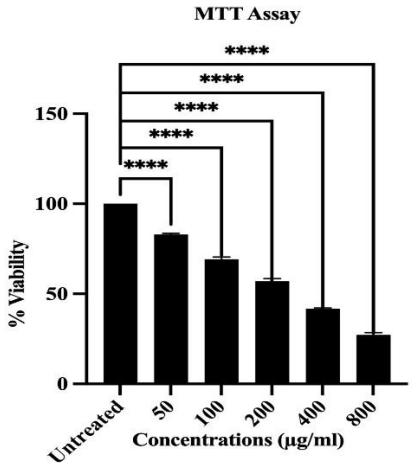

Figure 2 Dosage-dependent effect of EEMK on EAC cells in MTT assay, Values are represented as mean ± SEM (n = 3).

Apoptosis induction assay

The efficacy of EEMK in inducing the nuclear alterations in EAC was investigated using DAPI. The results demonstrated distinctive nuclear changes in response to EEMK treatment as the cells displayed deviations from the normal nuclear morphology, suggesting EEMK induces structural modifications in the nuclei of EAC. These alterations were particularly pronounced at a 1C50 concentration of 265 µg/ml, correlating with inhibited cell growth observed in the previous assays (Figure 3). The observed nuclear damage underscores the potential cytotoxic effects of EEMK, suggesting interference with cellular processes involved in maintaining cellular integrity.

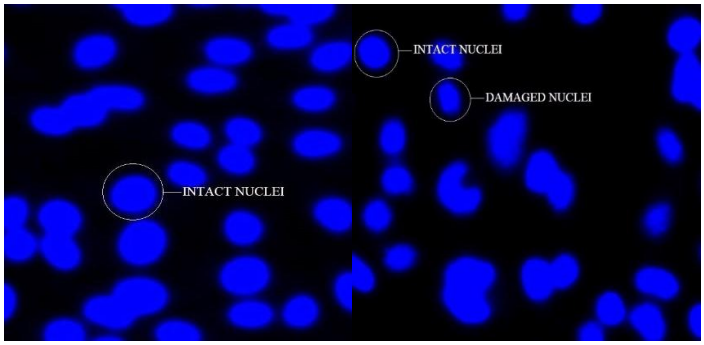

Figure 3 Alteration in the nuclear morphology of EA carcinoma cells induced by EEMK treatment

Acute oral toxicity

The acute oral toxicity assessment of EEMK indicated a favorable profile, with no observed toxic symptoms or mortality at the administered doses. The LD50 value was found to be greater than 2000 mg/kg body weight, highlighting the extract's relatively low acute toxicity indicating a significant margin of safety for oral consumption. The absence of adverse effects within the observation period demonstrates the potential of the extract for safe administration. Therefore, the dosage corresponding to $1/5^{th}$ and $1/10^{th}$ of the lethal dose was selected for the subsequent *in-vivo* antitumor activity.

Anti-tumor activity by EAC carcinoma model

In the present study, to study the antitumor activity of EEMK, the following parameters were evaluated:

Hematological parameters

In this study, we examined hematological parameters, including WBC, RBC, and HB counts, to gain insights into the influence of EEMK on immune response modulation myelosuppression and anaemia which are significant side effects of cancer chemotherapy. Our analysis revealed a distinct pattern across experimental groups, with a significant ($P < 0.0001$) increase in WBC count and a significant ($P < 0.0001$) decrease in RBC and HB counts in the disease control (DC) animals compared to the normal control (NC) group. Notably, EEMK at 400mg/kg exhibited a trend toward lower WBC counts almost similar to standard drug 5-Flurouracil, suggesting a potential impact on immune response modulation. Furthermore, the EEMK-treated groups restored RBC and HB counts to nearly normal, possibly affecting hematopoietic processes. These findings suggest that EEMK may have a beneficial influence on hematological parameters and immune responses, as shown in Figure 4.

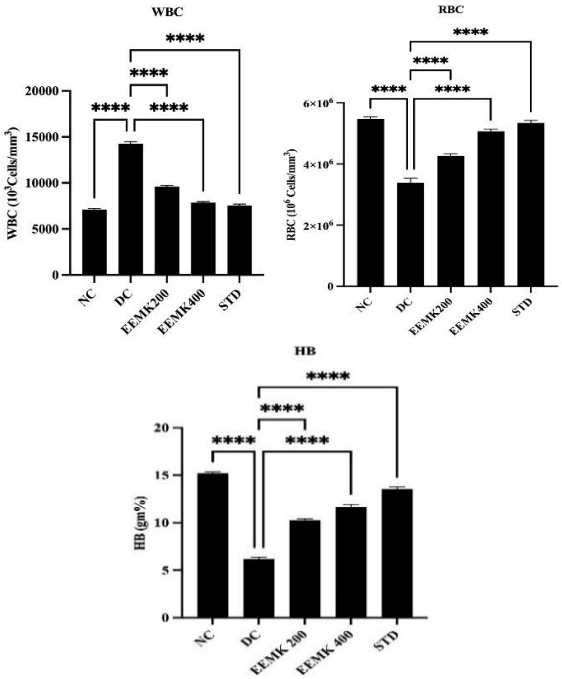

Figure 4 WBC, RBC, and HB Counts based on hematological parameters
Values are expressed as a mean of 6 animals ± SEM. All values ****P < 0.0001 significant compared to the EAC Control group

Tumor cell viability

The differentiation between the viable and non-viable cells within the tumor microenvironment serves as a crucial indicator of treatment efficacy. The ability to induce cell death selectively in the tumor cells without damaging the healthy cells is the distinctive trait that should be exhibited by therapeutic agents. The DC group exhibited substantial cell presence with a pronounced proportion of non-viable cells (Figure 5). This observation aligns with the disease's pathogenesis where the tumor cells evade apoptosis resulting in an accumulation of non-viable cells. The EEMK administration at both 200mg/kg and 400 mg/kg doses induced significant ($p<0.0001$) alterations in the tumor cell composition. It is observed that the EEMK-treated groups displayed a reduced viable cell population as compared to the DC group, signifying the potential of EEMK to impede the proliferation of the tumor cells. Concurrently, there was an increase in the presence of non-viable cells, indicating a potential shift towards apoptosis.

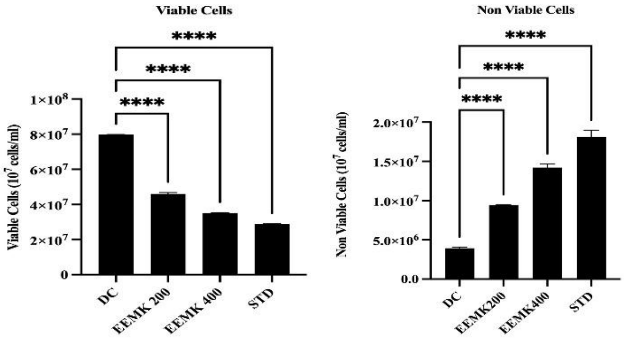

Figure 5 Effects of EEMK on tumor cell viability
Values are expressed as a mean of 6 animals ± SEM. All values are ****P < 0.0001 significant compared to (DC) EAC Control group

Effect of EEMK on tumor characteristics

The alterations in the packed cell volume, tumor volume, and change in body weight at a dose of 400mg/kg provide a holistic perspective on the impact of EEMK on tumor dynamics. The aggressive nature of the tumor cells can be seen clearly in the DC group, wherein the above-mentioned parameters were considerably elevated. A significant ($P < 0.0001$) decline in the packed cell volume following the administration of EEMK (both at 200 and 400 mg/kg) highlights the ability of the extract to attenuate the tumor associated with limiting the growth of the tumor. Similar trends were observed in tumor volume ****P < 0.0001, **P < 0.01 at 200 and 400 mg/kg respectively and tumor weight at dose 400mg/kg where EEMK administration led to an appreciable reduction in tumor volume and weight (Figure 6).

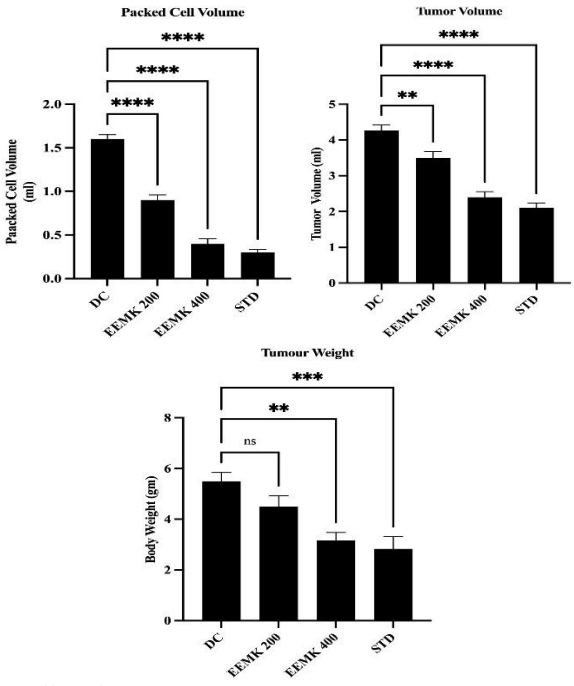

Figure 6: Effect of EEMK treatment on PCV, TV, and Body weight
Values are expressed as a mean of 6 animals ± SEM. ****P < 0.0001, ***P < 0.001, **P < 0.01 significant compared to EAC Control group, ns-non significant

Survival analysis

Evaluating the impact of the therapeutic intervention on the survival outcome is fundamental in antitumor research. The mean survival time (MST) and increase in life span (%ILS) are important parameters to assess the efficacy of therapeutics in prolonging survival. In the present study, MST and %ILS were not applicable for the normal group, given the absence of treatment, while the DC group exhibited MST of 20 days, reflecting the untreated course of the disease. The treatment with EEMK at both 200 mg and 400 mg doses recorded impressive prolongations in survival. There was a 60% increase in MST (32 days) in the 200mg group and a 95% increase (39 days) in the 400 mg group. The significant extension in survival recorded in the present study underscores the potential of EEMK to restrain tumor growth and enhance overall longevity (Table 1).

Table 1 Impact of EEMK treatment on the survival time

Groups	Normal	DC	EEMK (200 mg/kg)	EEMK (400mg/kg)	STD (20mg/kg)
MST(Days)	0	20±1.19	32±1.08	39±0.5	41±1.5
%ILS	--	--	60	95	105

Values are expressed as a mean of 6 animals ± SEM. ****P < 0.0001, significant compared to the EAC Control group

Biochemical parameters

Serum glutamate pyruvate transaminase (SGPT) and Serum glutamate oxaloacetate transaminase (SGOT) are key indicators of liver health, reflecting the release of these enzymes into the blood stream due to cellular damage. The normal group exhibited normal ranges of SGPT and SGOT while the DC exhibited significantly elevated levels of SGPT (216.58 IU/L) and SGOT (93.40 IU/L), indicating cellular damage and impaired hepatic function due to the tumor growth **(Gowda *et al*., 2022)**. Upon the administration of EEMK at both 200 mg and 400 mg dose there was a significant decrease ($p<0.0001$) in the SGPT and SGOT levels. The EEMK200 showed decrease SGPT (107.25 IU/L) and SGOT (73.37 IU/L) levels, while the EEMK400 group exhibited further reductions in the SGPT (61.88 IU/L**)** and SGOT (64.55 IU/L. These results imply potential mitigation of liver damage suggesting the hepatoprotective effect. (Figure 8).

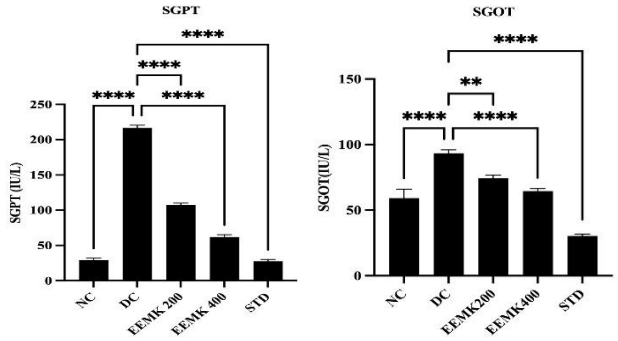

Figure 7 Effect of EEMK on SGPT and SGOT
Values are expressed as a mean of 6 animals ± SEM.****P < 0.0001, **P < 0.01 significant compared to the EAC Control group.

DISCUSSION

Cancer remains a significant global health challenge, requiring multifaceted strategies for effective management and intervention. The increasing burden of cancer emphasizes the need to explore novel therapeutic approaches that provide enhanced disease control while minimizing the adverse effects associated with treatments **(Chhikara & Parang, 2023)**. This study focuses on the potential anti-cancer properties of *Manilkara kauki* (also known as Caqui), a tropical plant recognized for its diverse range of bioactive compounds exhibiting promising antioxidant and ant tyrosinase activities **(T. K., 2012; Khare, 2007)**.

The primary objective of this investigation was to comprehensively examine the cytotoxic and antitumor effects of *Manilkara kauki* using a series of *in vitro* and *in vivo* analyses. Conventional strategies for cancer management, although varying in effectiveness, often entail significant limitations, including unfavourable side effects and the development of resistance. As a result, alternative therapies derived from natural sources like plants have gained prominence due to their capacity to provide targeted and well-tolerated interventions.

The *in vitro* analysis conducted in this investigation encompassed a thorough and multifaceted evaluation of *Manilkara kauki's* influence on EA carcinoma cells. To establish a comprehensive grasp of the potential mechanisms underlying the plant extract's actions, two distinct assays namely, the Trypan Blue assay and the MTT assay were employed. The Trypan Blue assay, serving as an evaluator of cell viability through the assessment of cell membrane integrity, played a pivotal role in revealing insights into the immediate cellular response to *Manilkara kauki* extract. This assay facilitated the observation of modifications in cell membrane integrity, a critical factor influencing cellular functionality and viability. Disruptions in cell membrane integrity are often associated with cell demise and can be attributed to various cytotoxic mechanisms, including apoptosis, necrosis, and autophagy **(Riss *et al*., 2004)**. By assessing the impact on cell membrane integrity, the Trypan Blue assay provided a glimpse into the extract's direct effects on EA carcinoma cells.

Complementing the insights obtained from the Trypan Blue assay, the MTT assay provided an alternative perspective by evaluating cellular metabolic activity. This assay relies on the reduction of MTT dye by metabolically active cells, allowing the quantification of viable cells based on their metabolic prowess. Cellular metabolism is intricately linked to cellular vitality and proliferation, with alterations often indicating shifts in cell signaling pathways, energy utilization, and nutrient availability **(Adan *et al*., 2016)**. Consequently, the MTT assay not only shed light on cellular survival capabilities but also hinted at potential mechanisms that underlie the extract's impact on vital biochemical pathways crucial for the survival of cancer cells. The observed alignment in the IC50 values resulting from both the Trypan Blue and MTT assays bears significant implications. The concurrence between these independent assays suggests that the cytotoxic effects of *Manilkara kauki* are uniform across diverse cellular pathways. This consistency bolsters the credibility of the findings and augments the overall dependability of the study's outcomes. It reinforces the notion that the discerned concentration threshold at which cytotoxic effects manifest is robust and dependable, establishing a sturdy groundwork for comprehending the extract's potential as an anticancer agent. Additionally, this convergence of outcomes across two distinct assays illustrates the extract's influence on EA carcinoma cells through multiple cellular mechanisms. While the Trypan Blue assay chiefly mirrors immediate cell death and membrane integrity, the MTT assay encapsulates broader metabolic activities and cellular well-being. The harmony between these assays heightens the study's credibility by validating that *Manilkara kauki's* effects extend beyond a singular pathway and encompass diverse facets of cellular function. The significance of the DAPI study in this mechanism behind the promising antitumor potential of the ethanolic extract of *Manilkara kauki* (EEMK). The DAPI staining fluorescence study provides crucial insights into how EEMK exerts its effects on cancer cells. Specifically, it demonstrates that EEMK triggers apoptosis in cancer cells, which is a programmed cell death process crucial for suppressing tumor growth. This finding suggests that EEMK may have a direct impact on cancer cell viability and proliferation, making it a potential candidate for further exploration in cancer therapy. The fluorescence study's results, in conjunction with the *in vitro* and *in vivo* assessments, reinforce the robustness of the overall findings. The convergence of data from different assays and experiments enhances the reliability of EEMK's antitumor effects.

The pivotal shift from *in vitro* experimentation to *in vivo* assessment constitutes a vital step in assessing the probable therapeutic significance of a natural compound, such as the extract from *Manilkara kauki.* This study's progression from cellular models to mice-bearing tumors provides a dynamic framework for probing the extract's implications within the intricate realm of biological processes. Administering EEMK to mice with tumors yielded noteworthy findings that affirm its marked antitumor efficacy. Particularly, the reduction observed in both tumor volume and body weight underscores the extract's potential to impede *in vivo* tumor expansion. This observation intimates that the extract could potentially regulate pivotal cellular pathways and mechanisms integral to tumor proliferation and enlargement, this could occur through inhibiting cellular division, instigating apoptosis, or disrupting pathways governing growth signaling.

The elevation in mean MST serves to further highlight the extract's therapeutic promise. The prolonged MST in treated mice not only indicates that EEMK hampers tumor progression but also exerts a systemic influence contributing to overall survival gains. This extension in MST implies that the extract might be influencing not solely the primary tumor but also metastatic dissemination and secondary growths, hinting at a multifaceted mode of operation. Arguably the most remarkable result manifests as the substantial increase in %ILS within treated mice. This metric summarizes the survival advantages conferred by the extract, thereby suggesting its potential to not only arrest tumor growth but also substantially enhance life's quality and duration. The notable %ILS values interest the extract's capacity to implicate critical processes beyond mere cytotoxicity potentially involving inhibiting angiogenesis, fostering immune responses, and molding the tumor microenvironment. These traits propose that the EEMK might be influencing various biochemical pathways intertwined with cancer progression.

The exact mechanism behind EEMK's cytotoxic impact is unknown. Anticancer activity is possibly associated with the presence of plant-derived terpenoids, flavonoids, and saponins present in the extract. Based on established data Terpenoids combat cancer by targeting numerous pathways such as mitochondrial death, PI3K/Akt, and NF-KB (**Kamran *et al*., 2022)**. Also, research suggests terpenoids could be employed as drugs to treat and prevent cancer. Flavonoids can trigger apoptotic and autophagic cell death and prevent cancer spread by regulating various signaling pathways **(Mir *et al*., 2024; Kopustinskiene *et al*., 2020).** Saponins cause apoptosis of tumor cells, which can reduce the side effects by preventing necrosis **(Man *et al*., 2010).**

The collective observed outcomes support the hypothesis that the antitumor effects of EEMK could originate from an array of intricate mechanisms. Its capability to check tumor expansion, prolong survival, and significantly enhance %ILS suggests its potential role in modulating angiogenesis, curbing growth-inducing signaling pathways, and impacting immune vigilance against tumor cells. These mechanisms coalesce to shape an environment less conducive to tumor survival and enlargement, ultimately ending in elevated survival prospects.

CONCLUSION

The present research investigated the antitumor potential of *Manilkara kauki* extract, employing a comprehensive methodology encompassing *in vitro* and *in vivo* evaluations. The findings underscore the extract's notable capacity to hinder tumor growth, evident through reductions in tumor volume and weight. Of equal significance, the extract exerted a substantial influence on overall survival, as indicated by a significant increase in MST and %ILS thereby suggesting its promise as a prospective therapeutic alternative in cancer treatment. The convergence of cytotoxicity data derived from both Trypan Blue and MTT assays increases the robustness of the outcomes, validating the extract's consistent impact on cell viability through diverse cellular pathways. Additionally, the extract's potential in mitigating treatment-related hepatic damage emphasizes its multifaceted attributes, extending beyond tumor-centric complications. While the findings present promise, exploring deeper into the underlying mechanisms becomes imperative to reveal the precise molecular targets and pathways influenced by the extract's bioactive components. Moreover, exploration of varying dosages and treatment protocols could yield valuable insights into optimizing its therapeutic efficacy. The outcomes of this study are worth continued inquiry into the intricate biochemical pathways modulated by the extract. Further research is needed to facilitate the identification of specific molecular targets for potential drug development. Further research work is in progress to isolate and characterize active constituents.

LIST OF ABBREVIATIONS

DAPI	4,6-diamidino-2-phenylindole dihydrochloride
DC	Disease Control
EAC	Ehrlich's ascites carcinoma
EEMK	Ethanolic extract of *Manilkara kauki*
HB	Haemoglobin
ILS	Increase in life span
NC	Negative control
MST	Mean survival time
PBS	Phosphate-buffered saline
RBC	Red blood cells
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
MST	Mean survival time
WBC	White blood cells
WHO	World Health Organization
STD	Standard (5 FU -5 Fluorouracil)

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