

## Anti-tumor effect of fruit rind of *Myristica malabarica* in an Ehrlich ascites carcinoma model

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### ABSTRACT

**Background:** Among the various modalities of anti-cancer treatment, cancer chemotherapy plays a very vital role. The alarming side effects being its main drawback leads to relentless research for newer agents. A new natural agent with promising anti-cancer properties from *in-vitro* studies leads to this study. Here we have evaluated the anti-tumor activity of a crude extract of fruit rind of *Myristica malabarica* in an Ehrlich ascites carcinoma model in mice.

**Methods:** A murine model of cancer was established with i.p. inoculation of Ehrlich Ascites carcinoma (EAC) cells; animals were divided into five groups (including normal control) to observe the inhibitory effect of a crude extract of the fruit rind of *Myristica malabarica/rampatri* (0-100mg/kg b.w. i.p.) as compared with methotrexate (0.4mg/kg bw., i.p.). Blood and ascitic fluid were collected on the 10<sup>th</sup> day for analysis.

**Results:** In the EAC model, there was an increase in tumor volume, tumor weight, and tumor packed cell volume, which was decreased by rampatri (50 and 100mg/kg bw) along with an increase in the mean survival time (MST). Rampatri caused minimal alterations in hematological parameters, renal functions remained unchanged but an increase in hepatic SGOT was demonstrated.

**Conclusions:** The crude extract of rampatri (containing Malabaricones) exhibited significant anti-tumor activity with minimal effect on hematological and renal functions.

**Keywords:** Anti-tumor, Ehrlich ascites carcinoma, Malabaricones, *Myristica malabarica*, Rampatri

### INTRODUCTION

There is an alarming increase in the incidence of cancer and the World Health Organization status reports in 2012 state an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths, compared with 12.7 million and 7.6 million, respectively in 2008 (<https://gco.iarc.fr/today/data-sources-methods>). The prevalence estimates for 2012 show that there were 32.6 million people (>15 years) who had had a cancer diagnosed in the previous five years. Though there is a plethora of cytotoxic drugs available for the treatment of

cancer, namely antibiotics, anti-metabolites, alkylating agents, inhibitors of formation of the mitotic spindle or inhibitors of topoisomerases, most of them are costly and have serious side effects that include myelosuppression and gastro-intestinal toxicity. It is relevant that 60% of currently used anticancer agents are derived from natural sources, including plants, marine organisms, and microorganisms. Considering the rich and diverse flora of India, the search for plant derived compounds is expected to yield important leads.

Malabaricones are diarylnonanoids containing constituents MAL-A, MAL-B, MAL-C and MAL-D sourced from the fruit rind of the plant *Myristica malabarica* popularly known as rampatri, Bombay mace or false nutmeg. Its pharmacological activities range from hepatoprotective, anti-ulcer, anti-leishmanial to anti-cancer activity.<sup>1-6</sup> The IC<sub>50</sub> in cancer cell lines was low as it ranged from 12.3±1.67 to 24±0.44μM<sup>4</sup> and accordingly, this study was undertaken to study its *in vivo* efficacy.

## METHODS

### Chemicals

All reagents were procured from Sigma-Aldrich (St Louis, Mo, USA) except the crude methanolic extract of rampatri.

### Animals

Adult male Swiss albino mice weighing 25-30g were acclimatized for one week in the institutional animal house at a temperature of 25-28°C, relative humidity of 55±2% and a 12hour light/dark cycle. The mice were grouped and housed in polyacrylic cages, with not more than six animals per cage, water and food pellets were provided *ad libitum*. The experimental protocols received prior approval from the institutional animal ethics committee in accordance with the ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Govt. of India.

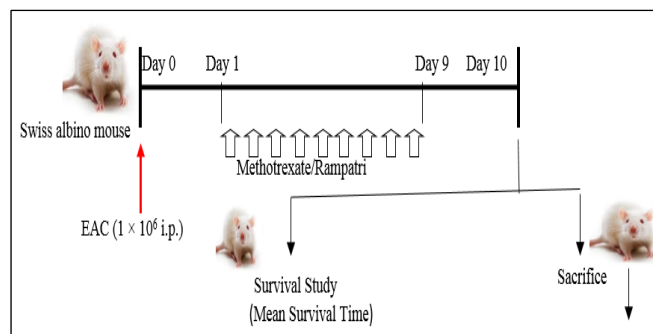
### Preparation of rampatri

A methanolic extract from the fruit rind of *Myristica malabarica* (Myristicaceae), popularly known as rampatri, Bombay mace or false nutmeg was prepared (1.0g/ml in DMSO) and stored at -20°C.<sup>5</sup> Just prior to administration, the extract was diluted in phosphate buffered saline (PBS, 20mM, pH 7.4).

### Development of an EAC model

Following centrifugation of mouse Ehrlich ascites carcinoma cells, the supernatant was removed and cell concentration was adjusted to 5×10<sup>6</sup> cells/mL. Under aseptic conditions, Swiss Albino mice (male, n=36) were injected these EAC cells (1×10<sup>6</sup> cells, i.p., 0.2ml/mouse).<sup>7</sup>

The control group (Group I, n=6) was randomly reserved before inoculation. Post 24hrs EAC transplantation, animals were then randomly divided into four groups (n=9), wherein group II, served as the EAC control group, group III received methotrexate (0.4mg/kg bw., i.p.) while groups IV and V received rampatri (50 and 100mg/kg bw., i.p) respectively for nine consecutive days (Figure 1) (MTX). In each group, 6 mice were euthanized after 24hrs of the last dose to evaluate tumor volume, tumor weight, and tumor packed cell volume, hematological and biochemical parameters, while three were kept for evaluating the mean survival time (MST).



EAC cells (1×10<sup>6</sup> cells, i.p., 0.2ml/mouse) were inoculated into 36 mice. Post 24 h EAC transplantation, animals were divided into four groups (n=9), wherein Group II, served as EAC control, Group III received methotrexate (0.4mg/kg bw., i.p.), Groups IV and V received rampatri (50 and 100mg/kg bw., i.p. respectively) for nine consecutive days as described in materials and methods.

**Figure 1: Study design for evaluation of anti-cancer effect of rampatri.**

### Tumor parameters

The effect of rampatri on tumor growth and host's survival time was assessed in terms of:

#### Tumor volume

The mice were dissected and ascitic fluid was collected from the peritoneal cavity. The volume was measured using a graduated centrifuge tube.

#### Tumor weight

The tumor weight was measured by taking the weight of the mice before (A) and after (B) collecting the ascitic fluid from the peritoneal cavity.

#### Tumor packed cell volume

After collecting the ascitic fluid, the tumor packed cell volume was determined by centrifugation (1000g x 5 minutes).

#### Determination of mean survival time and percentage increase in life span

The mortality was monitored by recording percentage increase in life span (% ILS) and mean survival time (MST) as follows:

$$\% \text{ ILS} = \left[ \frac{\text{Mean survival time of treated group}}{\text{Mean survival time of control group}} - 1 \right] \times 100$$

#### Estimation of hematological, renal and liver function tests

Blood was collected 24hrs after the last dose, and 18 h of fasting for estimation of hemoglobin (Hb), RBC and WBC

count, platelet and lymphocyte counts, hematocrit, mean corpuscular volume, in a coulter counter. Urea and creatinine were estimated for evaluation of renal functions, while liver functions were evaluated in terms of serum glutamate oxaloacetate transaminase (SGOT) and glutamate pyruvate transaminase (SGPT), using commercially available kits.

### Statistical analysis

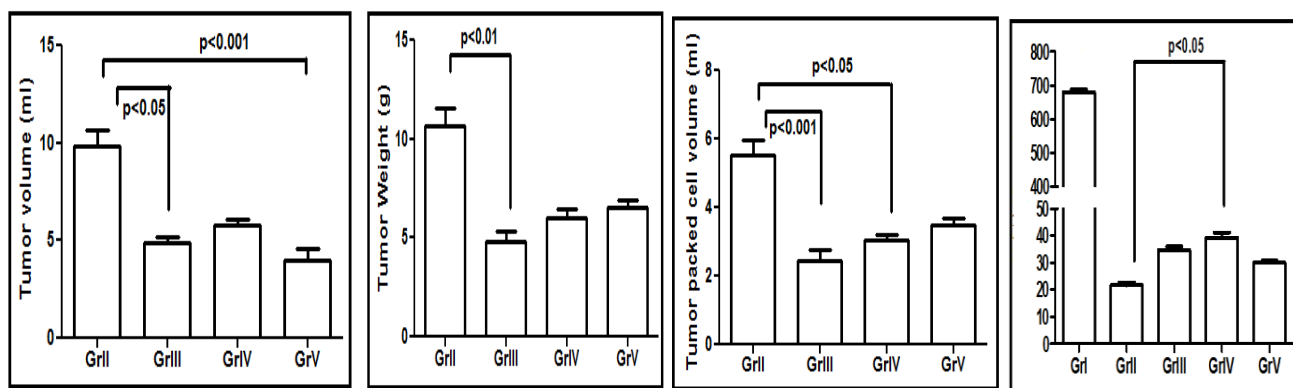
All data were expressed as mean $\pm$ SEM for the laboratory and tumor parameters and survivability. Statistical analysis was evaluated by one-way ANOVA followed by Dunnett's post hoc test using Graph Pad Prism software,

version 5(Graph Pad Software Inc., San Diego, CA, USA);  $p < 0.05$  were considered as statistically significant.

## RESULTS

### Tumor volume

In the EAC control (Group II), the tumor volume increased to  $9.85 \pm 0.84$  ml which in the presence of MTX (Group III) led to a significant 2.01 fold decrease to  $4.88 \pm 0.27$  ml ( $p < 0.05$ , Figure 2A). Similarly, rampatri (50 and 100 mg/kg bw., groups IV and V) showed a dose dependent decrease, as the tumor volume decreased by 1.69 and 2.4 fold to  $5.80 \pm 0.29$  and  $4.00 \pm 0.55$  ml ( $p < 0.001$ ) respectively (Figure 2A).



Swiss albino mice (Group I), were then inoculated with  $1 \times 10^6$  EAC cells (Group II) and treated with Methotrexate (0.4 mg/kg b.w., Group III) or rampatri (50 or 100 mg/kg b.w., Groups IV or V) for 9 consecutive days as described in Materials and methods. Data are expressed as the mean  $\pm$  SEM, (n= 6) in each experimental group.

**Figure 2: Effect of rampatri on tumor characteristics. (A) Tumor volume, (B) Tumor weight, (C) Tumor packed cell volume, (D) Mean survival time (MST) were evaluated in Swiss albino mice.**

### Tumor weight

A substantial increase in tumor weight was observed in the EAC animal model being  $10.63 \pm 0.95$  g which decreased significantly by 2.06 fold with the addition of MTX to  $4.80 \pm 0.56$  g ( $p < 0.01$ , Figure 2B). Rampatri (50 and 100 mg/kg bw. Groups IV and V) also demonstrated a 1.76 and 1.62 fold decrease in tumor weight to  $6.02 \pm 0.40$  and  $6.54 \pm 0.35$  g respectively (Figure 2B).

### Tumor packed cell volume

Following the addition of MTX (Group III), the increased tumor packed cell volume in the EAC model ( $5.51 \pm 0.47$  ml) substantially decreased by 2.2. fold to  $2.46 \pm 0.29$  ml,  $p < 0.001$ , Figure 2C). Rampatri (50 and 100 mg/kg bw. Groups IV and V) also decreased the tumor packed cell volume by 1.8 fold ( $3.04 \pm 0.18$  ml,  $p < 0.05$ , Figure 2C) and 1.5 fold ( $3.50 \pm 0.19$  ml) respectively (Figure 2C).

### Increase in life span

The Mean survival Time (MST) was decreased in the EAC control group ( $21.67 \pm 1.11$  days) in comparison with normal Swiss albino mice which remained alive for at least 6 months after the study was completed, indicating establishment of the tumor model (Figure 2D). The MST increased by 1.59 fold following treatment with MTX to  $34.67 \pm 1.28$  days. Similarly, treatment with rampatri (Groups IV and V) increased the MST by 1.81 ( $p < 0.05$ ) and 1.38 fold respectively (Figure 2D).

### Hematological parameters

In the tumor model, there was a significant decrease in the RBC and lymphocyte count, hemoglobin, hematocrit, mean corpuscular volume. Treatment with rampatri or MTX restored these parameters except for the lymphopenia which persisted (Table 1).

**Table 1: Effects of rampatri on hematological parameters of EAC model.**

Parameters	Group (drug, mg/kg b.w., i.p.)				
	Group I (normal)	Group II (No drug)	Group III (MTX, 0.4 mg/kg b.w.)	Group IV (Rampatri, 50 mg/kg b.w.)	Group V (Rampatri, 100mg/kg b.w.)
WBC ( $\times 10^3/\mu\text{l}$ )	11.25 $\pm$ 0.93	23.20 $\pm$ 1.79*	10.87 $\pm$ 0.77#	13.25 $\pm$ 0.77#	12.07 $\pm$ 0.67#
RBC ( $\times 10^6/\mu\text{l}$ )	8.53 $\pm$ 0.87	4.49 $\pm$ 0.24*	6.96 $\pm$ 0.76#	7.47 $\pm$ 0.33#	7.42 $\pm$ 0.10#
Hb (g/dl)	13.00 $\pm$ 1.31	8.30 $\pm$ 0.30*	10.92 $\pm$ 0.75#	12.55 $\pm$ 0.24#	11.78 $\pm$ 0.41#
HCT (%)	42.95 $\pm$ 1.45	26.15 $\pm$ 1.28*	32.07 $\pm$ 1.17*#	34.02 $\pm$ 1.22*#	35.55 $\pm$ 1.34*#
MCV (fl)	51.80 $\pm$ 1.08	56.88 $\pm$ 1.37	47.08 $\pm$ 1.07#	46.15 $\pm$ 1.22*#	47.23 $\pm$ 1.35#
PLT ( $\times 10^3/\mu\text{l}$ )	359.8 $\pm$ 46.61	344.2 $\pm$ 55.57	276.2 $\pm$ 37.62	482.0 $\pm$ 41.39 <sup>\$</sup>	527.3 $\pm$ 56.90 <sup>\$</sup>
LYM (%)	76.48 $\pm$ 3.81	28.23 $\pm$ 1.71*	32.1 $\pm$ 3.30*	35.40 $\pm$ 3.41*	36.78 $\pm$ 2.67*

\*p<0.05 compared with Group I; #p<0.05 compared with Group II; \$p<0.05 compared with Group III

**Table 2: Effects of rampatri on hepatic and renal functions of EAC bearing male Swiss Albino mice.**

Parameters	Group I (normal)	Group II (No drug)	Group III (MTX, 0.4mg/kg b.w.)	Group IV (Rampatri, 50mg/kg b.w.)	Group V (Rampatri, 100mg/kg b.w.)
Urea (mg/dl)	50.17 $\pm$ 3.29	33.15 $\pm$ 1.49	31.00 $\pm$ 1.93	37.83 $\pm$ 1.13	33.83 $\pm$ 1.60
Creatinine (mg/dl)	0.53 $\pm$ 0.04	0.37 $\pm$ 0.10	0.58 $\pm$ 0.09	0.45 $\pm$ 0.02	0.36 $\pm$ 0.01
SGOT(U/L)	40.83 $\pm$ 3.80	51.92 $\pm$ 7.08	76.33 $\pm$ 7.03	270.2 $\pm$ 6.90*# <sup>\$</sup>	276.3 $\pm$ 21.22*# <sup>\$</sup>
SGPT(U/L)	42.17 $\pm$ 5.82	43.00 $\pm$ 2.33	91.50 $\pm$ 9.55*	44.83 $\pm$ 1.95#	42.17 $\pm$ 2.54 <sup>\$</sup>

\*p<0.05 compared with Group I; #p<0.05 compared with Group II; \$p<0.05 compared with Group III

### Effect of rampatri on renal and hepatic functions

Levels of urea and creatinine remained unchanged across all groups (Table 2). The serum SGOT in the EAC control group (group II) was comparable with group I, but both doses of rampatri (group IV and V) significantly increased the serum SGOT levels (p<0.05, Table 2). In the EAC control group, serum SGPT levels were unchanged (Table 2), but MTX (group III) significantly increased the SGPT levels by 1.3 fold as compared to the EAC treated group (p<0.05, Table 2), but was not observed with rampatri.

## DISCUSSION

The anticancer properties of malabaricones derived from rampatri have been demonstrated in cancer cell lines and the cytotoxicity attributed to its ability to trigger a redox imbalance, possibly secondary to its Cu (II)-dependent nuclease property.<sup>4,6,8,9</sup> The maintenance of redox homeostasis within cells is essential for the survival of a cell and in the perspective of cell metabolism, cancer cells adapt themselves to handle the redox imbalance by developing strategies that make them become insensitive to stress inducers such as chemotherapy and radiation.<sup>10</sup> However, the excessive induction of ROS by compounds such as malabaricones have been proposed reactive oxygen species to be potential therapeutic tools against cancers.<sup>4,8-10</sup>

The pathogenesis of malignant ascites involves injury to the peritoneal serosa by malignant cells leading to increased vascular permeability, edema formation, cellular

migration and progressive ascitic fluid accumulation. This ascitic fluid serves as a direct nutritional source for tumor cells allowing for tumor growth. This was demonstrated in the EAC model where the increase in tumor parameters e.g. tumor volume, tumor weight and tumor packed cell volume in EAC bearing mice was demonstrated (Figure 2). Importantly, the reduction of these tumor parameters by rampatri along with prolongation of the life span of tumor bearing animals endorsed the anti-tumor activity of rampatri in an animal model, endorsing *in vitro* studies performed in cell lines.<sup>4,8,9</sup>

The major drawback of cancer chemotherapy includes myelosuppression and anemia, and the ability of rampatri to improve the hematological profile suggested that the compound did not adversely impact on hematopoiesis, and was in concordance with toxicity studies that demonstrated it was non-toxic till 2000mg.<sup>11-14</sup> Furthermore, the unchanged renal functions endorsed the minimal toxic effect of rampatri.

However, rampatri caused a significant increase in SGOT perhaps secondary to its pro-oxidant property. Taken together, rampatri displayed promising *in vivo* antitumor activity and minimal toxicity endorsing further *in vivo* studies be undertaken with its active phytoconstituents, namely malabaricones.<sup>4,8,9</sup>

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee (IAEC, 544/PO/c/02/CPCSEA)

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