

## RESEARCH COMMUNICATION

**Anti-Tumour Activity of *Ruta Graveolens* Extract****K C Preethi, Girija Kuttan, Ramadasan Kuttan\*****Abstract**

An extract of *Ruta graveolens* was found to be cytotoxic to Dalton's lymphoma ascites (DLA), Ehrlich ascites carcinoma (EAC) and L929 cells in culture (IC100 = 16mg/ml) and also to increase the lifespan of tumour bearing animals. The extract further decreased solid tumours developing from DLA and EAC cells when given simultaneously with elongation of the lifespan of tumour-bearing animals. A homeopathic preparation of *Ruta graveolens* (200c) was equally effective. Neither was effective for reducing already developed tumours. The *Ruta graveolens* extract was found to scavenge hydroxyl radicals and inhibit lipid peroxidation at low concentrations. However, at higher concentrations the extract acted as a prooxidant as inhibition of lipid peroxidation and scavenging of hydroxyl radical was minimal. These data indicates that the prooxidant activity of *Ruta graveolens* may be responsible for the cytotoxic action of the extract and its ability to produce tumour reduction.

**Key Words:** *Ruta graveolens* - anti tumour drugs - antioxidants - prooxidants - herbal drugs.

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**Introduction**

One of the major limitations in the currently available treatment modalities for cancer is their side effects. Chemotherapy has been shown to produce severe neutropenia, which can lead to opportunistic infections, which may even lead to death. Hence alternate treatment for cancer is being tested and plants and plant derived products are being tried for the purpose. Information on the ethnopharmacologic use of plants has given important lead in the cancer drug development.

*Ruta graveolens*. L (Family Rutaceae) is a medicinal plant, used since time immemorial. Traditionally Rue is considered to be emmenagogue, ecbolic, anthelmintic and antispasmodic. It has been approved by Food and Drug Administration (FDA) as a flavouring agent. Anti-inflammatory (Raghav et al., 2005) anti fungal (Oliva et al., 2003) antibacterial (Ojala et al., 2000) and hypotensive (Chiu and Fung, 1997) activities of *Ruta graveolens* have been scientifically proven but at higher concentrations it has been reported to exhibit toxicity (Agraa and Balwi, 2002).

Use of *Ruta graveolens* against intracranial cysticercosis has been reported (Banerji and Banerji, 2001). Recently Pathak et al., (2003) showed that homeopathically potentiated *Ruta* induces cell death in brain cancer cells. Telomere dynamics, mitotic catastrophe and apoptosis have been shown to be the possible mechanisms. However, no systematic effort has been taken to establish antitumour activity of *Ruta graveolens* in animal models. In the present

study we have checked the antitumour activity of *Ruta graveolens* against ascites and solid tumour models. We also assessed the effects of potentiated *Ruta graveolens* and possible mechanisms of toxicity were investigated.

**Materials and Methods***Chemicals*

Deoxyribose was purchased from Sisco Research Laboratories Pvt, Ltd. Mumbai. Thiobarbituric acid and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Himedia laboratories, Mumbai. MTT was purchased from Sigma Chemicals; U.S.A. Foetal Calf Serum was purchased from Biological Industries, Israel. All other chemicals and reagents used were of analytical grade.

*Cells*

L929 cells were purchased from the National Facility for Animal Tissue and Cell Culture, Pune. Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells are being maintained in our laboratory in the mouse peritoneal cavity.

*Drug preparation*

*Ruta graveolens* was purchased from a local market. A voucher specimen with number Ru02 was deposited at the herbarium of Amala Cancer Research Centre, Amala Nagar, Kerala, India after authentication. Homeopathic dilution of *Ruta* was procured from Willmar Schwabe, Germany. Fresh

leaves and tender stem (100gms) was blended to a paste in a mixer and then extracted first with 1000ml of 75% methanol by soaking overnight with occasional stirring. The supernatant was decanted and the residue was again extracted with 500ml 75% methanol. The supernatant were pooled and concentrated in a vacuum evaporator at 42°C. The concentrated extract was redissolved in water for antioxidant studies. For cytotoxicity and in vivo studies, the extract was redissolved in minimum amount of alcohol and was then made up to the required volume in 0.1% carboxy methyl cellulose. In the case of homeo preparation, 10µl of Ruta 200c was diluted to 0.1ml in water and used for animal administration.

#### HPTLC analysis

HPTLC analysis of the crude extract of *Ruta graveolens* was done using Silica GF 254 plates with the solvent system toluene: ether (1:1) saturated with 10% acetic acid. The detection wavelength of the chromatogram was at 356 nm. Rutin was used as the standard.

#### Experimental animals

Female BALB/c mice (20-25 gm) were obtained from Small Animal Breeding Station, Mannuthy, Thrissur. They were kept in well-ventilated cages with normal mouse chow (Sai Durga Food and Feed, Bangalore) and water *ad libitum*. All animal experiments were conducted with the permission of the Institutional Ethics Committee.

#### Determination of the in vitro cytotoxic activity of *Ruta graveolens* to DLA and EAC cells.

DLA cells and EAC cells were aspirated from the peritoneal cavity and washed 3 times with PBS. 1 million cells were incubated with various concentrations of the extract (400µg; 800µg; 1.6mg; 4mg; 8mg; 16mg/ml) in a total volume of 1ml for 3 hours at 37°C. After incubation, the viability of the cells was determined by the trypan blue exclusion method (Talwar, 1978).

#### Determination of the cytotoxicity of *Ruta graveolens* to L929 cells in culture.

L929 cells (5000 cells/ well) were plated in 96 well flat bottom titre plates. After 24hr of incubation at 37°C in 5% CO<sub>2</sub> atmosphere, different concentrations of *Ruta graveolens* extract (400µg; 800µg; 1.6mg; 4mg; 8mg; 16mg/ml) were added and further incubated for 48 hrs. 20µl MTT (5mg/ml) was added 4hr before the completion of incubation (Cole, 1986; Campling et al., 1991). The plates were centrifuged and the supernatant removed and then 100µl DMSO was added and the intensity of the blue colour was read at 570nm using ELISA plate reader.

#### Determination of the effect of *Ruta graveolens* on ascites tumor bearing animals.

Both DLA and EAC cell lines were used for the experiment. Ascites tumors were induced in ten groups (6 animals/group) of BALB/c mice by injecting 1 million cells

/animal in the peritoneal cavity. Drug administration was started after 24hr of tumour inoculation and continued for 5 consecutive days. The animals were grouped as follows:

Group I: DLA cells alone.

Group II: DLA cells + 400mg/kg b.wt extract in CMC (i.p).

Group III: DLA cells + 200 mg/kg b.wt extract (i.p)

Group IV: DLA cells + 80 mg/kg b.wt extract (i.p)

Group V: DLA cells +10µl Ruta 200c diluted to 0.1ml (oral)

Group VI: EAC cells alone.

Group VII: EAC cells + 400mg/kg b.wt extract (i.p)

Group VIII: EAC cells + 200 mg/kg b.wt extract (i.p)

Group XI: EAC cells + 80 mg/kg b.wt extract (i.p)

Group X: EAC cells + 10µl Ruta 200c in 0.1ml (oral).

The death pattern of the animals due to tumour burden was noted everyday and the percentage of increase in lifespan was calculated using the formula  $T-C/C \times 100$  were 'T' and 'C' are the number of days that treated and control animals survived respectively (Kuttan et al., 1985).

#### Determination of the effect of *Ruta graveolens* on the solid tumour development.

Dalton's lymphoma ascites (DLA) cells and Ehrlich ascites carcinoma (EAC) cells ( $1 \times 10^6$  cells/ animal) were injected subcutaneously on the right hind limb of 10 groups (6 animals/group) of BALB/c mice as for the ascites case.

At 24hrs after tumour inoculation, different doses of the drug was given and continued for 10 consecutive days. Initial diameter of the hind limb was noted using vernier calipers. From 7th day onwards the tumour diameter was measured every 3rd day and recorded up to 46 days. The tumour volume was calculated using the formula  $V = \frac{4}{3} \pi r^2 r^2$  (Kuttan et al., 1985). The survival of the animals was recorded for up to 150 days.

#### Determination of the effects of *Ruta graveolens* on the development of solid tumours.

Dalton's lymphoma ascites (DLA) cells and Ehrlich ascites carcinoma (EAC) cells ( $1 \times 10^6$  cells/ animal) were injected subcutaneously on the right hind limb of 8 groups (6 animals/group) of BALB/c mice.

Group I: DLA cells alone.

Group II: DLA cells + 400mg/kg b.wt extract in CMC (i.p).

Group III: DLA cells + 200 mg/kg b.wt extract (i.p)

Group IV: DLA cells +10µl Ruta 200c in 0.1ml (oral)

Group V: EAC cells alone.

Group VI: EAC cells + 400mg/kg b.wt extract (i.p)

Group VII: EAC cells + 200 mg/kg b.wt extract (i.p)

Group VIII: EAC cells + 10µl Ruta 200c in 0.1ml (oral).

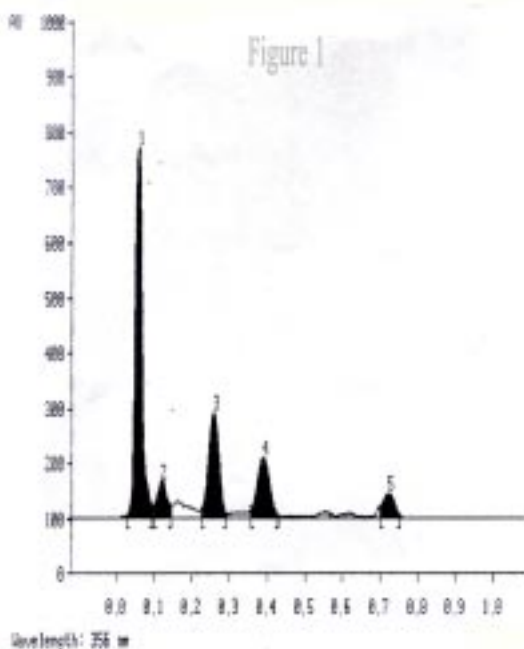
The treatment with the drug was started after the tumour size reached 1cc. Further the animals were treated with drug for 20 consecutive days. The tumour diameter was recorded there after on every 3rd day and the volume was calculated as above.

**Determination of in vitro hydroxyl radical scavenging activity.**

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compound for hydroxyl radicals generated from the  $Fe^{3+}$ /ascorbate/EDTA/ $H_2O_2$  system. The hydroxyl radical attacks deoxy ribose, which results in thiobarbituric acid reacting substance (TBARS) formation (Elizabeth and Rao, 1990). The reaction mixture contained deoxyribose (2.8mM),  $FeCl_3$  (0.1mM), EDTA (0.1mM)  $H_2O_2$  (1mM), ascorbic acid (0.1mM),  $KH_2PO_4$ -KOH buffer (20mM pH 7.4) and various concentrations of the extract in a final volume of 1ml. The reaction mixture was incubated for one hour at 37°C. Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated.

**Determination of lipid peroxidation.**

Reaction mixture (0.5 ml) containing rat liver homogenate (0.1 ml, 25% w/v) in Tris-HCl buffer (40 mM, pH 7.0), KCl (30mM), ferrous ions (0.16 mM) and ascorbic acid (0.06mM) were incubated for one hour at 37°C in the presence and absence of the extracts. The lipid peroxide formed was measured by TBARS formation (Ohkawa et al., 1979). Incubation mixtures (0.4ml) were treated with sodium dodecyl sulphate (SDS-8.1%, 0.2ml), thiobarbituric acid (TBA-0.8%, 1.5ml) and acetic acid (20%, 1.5ml pH-3.5). The total volume was then made up to 4ml with distilled water and kept in a water bath at 100°C for one hour. After cooling, 1ml of distilled water and 5ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and vortexed. After centrifugation, the absorbance of the organic layer was measured at 532nm. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compound with those of control, not treated with the extract.



**Figure 1. HPTLC Finger print of *Ruta graveolens* Extract**

**Table 1. Cytotoxicity of *Ruta graveolens* to Cells**

Concentration/ml	DLA	EAC	L929
400µg	1%	0%	3.0%
800µg	1%	1%	10.9%
1.6mg	2%	1%	33.3%
4.0mg	6%	8%	63.1%
8.0mg	44%	98%	82.5%
16.0mg	100%	100%	100%

**Statistical analysis**

The results are expressed as mean  $\pm$  S.D. Statistical evaluation of the data was carried out by one way ANOVA followed by Dunnet's test (post-hoc) using In Stat 3 software package.

**Results****HPTLC Pattern**

The fingerprint obtained after HPTLC analysis of *Ruta graveolens* extract is given in Figure 1.

**Cytotoxicity of *Ruta graveolens* towards DLA, EAC and L929 cells.**

*Ruta graveolens* was found to be cytotoxic towards DLA and EAC cells only at higher concentration. The 100% cytotoxicity was attained only at 16mg/ml. It was cytotoxic to L929 and showed 100% cytotoxicity at the concentration of 16mg/ml (Table 1).

**Effect of *Ruta graveolens* on lifespan of ascites tumour bearing animals.**

Lifespan of ascites tumour bearing animals induced by DLA cells was found to be increased by *Ruta graveolens* treatment. In 400mg/kg b.wt group the lifespan was increased by 21.6% and in 200mg/kg b.wt group the increase was 43.2%. In 80mg/kg b.wt group there was a significant increase in lifespan ( $p < 0.001$ ) of 66.5%. Administration of *Ruta* 200c (10µL) (Homeo preparation) increased the lifespan of ascites tumour bearing animals by 47.7% (Table 2).

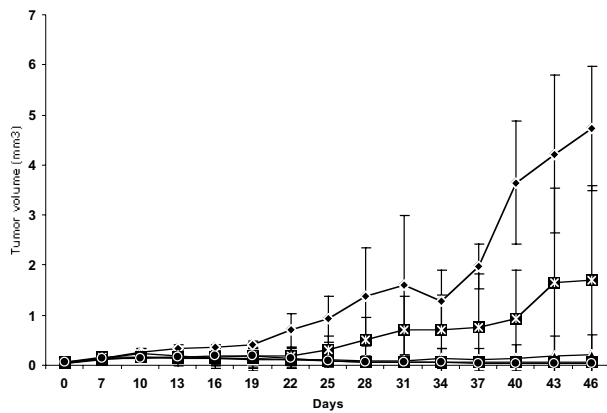
There was a significant increase in lifespan of EAC cell induced tumour bearing animals after treatment with *Ruta graveolens* extract. In 400mg/kg b.wt group the increase of lifespan was 45.0% ( $p < 0.05$ ) and in 200mg/kg b.wt group

**Table 2. Effect of *Ruta graveolens* on Lifespan of Ascites Tumour-bearing Animals**

Treatment	DLA cells		EAC cells	
	Survival <sup>1</sup>	Increase	Survival	Increase
Tumour (T)	18.5 $\pm$ 1.9	-	18.0 $\pm$ 1.8	-
T + 80mg	30.8 $\pm$ 2.9***	66.5%	25.0 $\pm$ 5.8**	38.9%
T+ 200mg	26.5 $\pm$ 7.7	43.2%	32.6 $\pm$ 7.3***	81.1%
T+ 400mg	22.5 $\pm$ 3.7	21.6%	26.1 $\pm$ 8.8*	45.0%
T + <i>Ruta</i> <sup>2</sup>	27.3 $\pm$ 6.9**	47.7%	27.0 $\pm$ 4.1*	45.9%

<sup>1</sup>Mean days, <sup>2</sup>200c (10µl/animal);

\*, \*\*, \*\*\*  $p < 0.01$ ,  $p < 0.005$  and  $p < 0.001$ , respectively



**Figure 2. Effect of *Ruta graveolens* on Solid Tumour Development from DLA cells**

the increase was 81.1% ( $p < 0.001$ ). In 80mg/kg b.wt group the increase in lifespan was found to be 38.9% ( $p < 0.01$ ) and in group getting *Ruta* 200c 10mL/animal, the increase was 45.9% ( $p < 0.05$ ) (Table 2). These results also indicated that *Ruta graveolens* extract increased the lifespan of tumour bearing animals but at higher concentration it produced decreased lifespan indicating possible tissue toxicity.

#### *Effect of Ruta graveolens on solid tumour induced by DLA cells with simultaneous drug treatment.*

A significant reduction of solid tumour was found in *Ruta graveolens* extract treated groups when compared with the control from 22nd day of tumour inoculation. On 34th day the tumour volume of the control animals without any drug treatment was found to be  $1.29 \pm 0.61 \text{ mm}^3$ , which was significantly higher compared to, treated groups  $0.08 \pm 0.03 \text{ mm}^3$  ( $p < 0.01$ ) (80mg/kg b. wt),  $0.14 \pm 0.2 \text{ mm}^3$  ( $p < 0.01$ ) (200mg/kg b.wt) and  $0.71 \pm 0.7 \text{ mm}^3$  (400 mg/kg b.wt). As shown in Figure 2 there was significant decrease in tumour volume in treated groups on other days as well. In *Ruta* 200c treated group tumour volume was found to be  $0.15 \pm 0.21 \text{ mm}^3$  ( $p < 0.01$ ).

#### *Effect of Ruta graveolens on solid tumour induced by EAC cells.*

Significant reduction in tumour was also found in EAC induced solid tumour animals treated with different concentrations of *Ruta graveolens* extract. On 34th day the mean tumour volume of the control animals were  $3.4 \pm 1.9 \text{ mm}^3$ . But in 80mg/kg b.wt, 200mg/kg b.wt, 400mg/kg b.wt the mean tumour volume were  $0.73 \pm 1.2 \text{ mm}^3$  ( $p < 0.01$ ),  $0.16 \pm 0.11 \text{ mm}^3$  ( $p < 0.01$ ), and  $1.52 \pm 1.9 \text{ mm}^3$  respectively. As in the case of ascites tumour, increased concentration of *Ruta* had an adverse effect in solid tumour reduction. Moreover, homeopathic dilution of *Ruta* significantly reduced the tumour volume.

Although a comparative reduction or slowing down of tumour development was found to occur in the animals treated after tumour development, a significant reduction was not seen in the case of both DLA and EAC cells (data not shown).

#### *Effect of Ruta graveolens on lifespan of solid tumour bearing animals. DLA cell derived solid tumours:*

The mean survival days were significantly increased by treatment with *Ruta graveolens*. In case of simultaneous drug treatment 2 animals died in 80mg/kg b.wt group and 1 animal in 200mg/kg b.wt group and 4 animals in 400mg/kg b.wt group within 150 days, and increase in the lifespan was 122.8% ( $p < 0.01$ ), 130.0% ( $p < 0.01$ ) and 63.2% respectively when compared to that of control which had an average lifespan of  $58.7 \pm 14.9$  days. In *Ruta* 200c treated group also only 1 animal died within 150 days and the increase in lifespan was 126.1% ( $p < 0.01$ ).

#### *EAC cell derived solid tumours:*

Mean survival days up to 150 days were significantly increased in treated groups. In 80mg/kg b.wt and 400mg/kg b.wt groups, 4 animals were died by 150 days and only 2 animals died in 200mg/kg b.wt group. In *Ruta* 200c only 1 animal was dead within 150 days. Increase in lifespan was 93.8% ( $p < 0.05$ ), 126.0% ( $p < 0.01$ ), 81.8% and 147.7% ( $p < 0.01$ ) for 80mg/kg b.wt, 200mg/kg b.wt, 400mg/kg b.wt and *Ruta* 200c respectively while in control group the average life span was found to be  $55.8 \pm 13.4$  days.

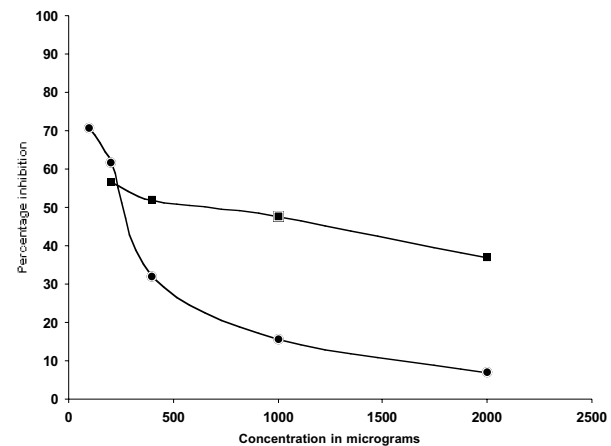
#### *Effect of Ruta graveolens on developed solid tumour bearing animals.*

Although a comparative reduction or slowing down of tumour development was found to occur in the treated groups, a significant reduction was not seen when the treatment was started 30 days after tumour induction (data not shown).

#### *Effect of Ruta graveolens on in vitro antioxidant system.*

At lower concentration *Ruta graveolens* extract was found to possess antioxidant activity while at higher concentration it did not. It inhibited 70% of hydroxyl radical at 100mg/ml where as at 2000mg/ml the inhibition was only 6.9%. This indicated that at higher concentration the extract produced a prooxidant activity (Figure 3).

Similarly at lower concentration extract inhibited lipid



**Figure 3. Effect of *Ruta graveolens* Extract on in vitro Lipid Peroxidation and Hydroxyl Radical Scavenging**

peroxidation effectively. At 100mg/ml concentration the inhibition was 56.6%. However it was found that *Ruta graveolens* extract at higher concentration did not inhibit the in vitro lipid peroxidation. At 2000mg/ml concentration it inhibited lipid peroxidation only by 21.2% only (Figure 4) which again indicate that at higher concentration, *Ruta graveolens* extract may have pro-oxidant activity.

## Discussion

*Ruta graveolens* was found to possess antitumour activity. It had a significant effect in increasing the lifespan of ascites DLA and EAC tumour bearing animals. It was also found to reduce the solid tumour in animal models. The survival rate of solid tumour bearing animals was also significantly increased by *Ruta graveolens* extract treatment. But it was found that the activity was not concentration dependent. When the higher concentration was used, the activity was found to be reduced than that of the lower concentration in both ascites and solid tumour model which may be due to the toxicity of *Ruta graveolens* extract which has been previously reported (Agraa and Balwi, 2002).

Interestingly it was found that the homeopathic dilution of *Ruta*, the *Ruta* 200c is also effective against ascites and solid tumour. Previous reports say that the homeopathic dilution of *Ruta* has anticlastogenic activity (Khuda-Bukhsh and Maity, 1990). In our laboratory the effect of homeopathic dilution of *Ruta* inhibited NDEA induced liver carcinogenesis as seen by enzyme parameters and histopathologic evaluation indicated that *Ruta* 200c is effective against chemical carcinogenesis in the liver.

In vitro experiments to check the antioxidant activity of *Ruta graveolens* revealed that at higher concentration, it acted as a pro-oxidant rather than an antioxidant. But at lower concentration *Ruta graveolens* extract was found to scavenge hydroxyl radical and inhibited lipid peroxidation. Prooxidants have the capability to change the redox potential in the cell and it is already reported that prooxidants have direct effect on mitochondrial permeability transition pore (Kushnareva and Sokolove, 2000). It activates the mitochondrial permeability transition pore and thus leads to apoptosis. *Ruta graveolens* extract was also found to induce apoptosis (unpublished data). So it might have action directly on DNA strands that induces cell death.

The *Ruta graveolens* was reported to have the constituents which include Flavonoids rutin, alkaloids quinolone, furoquinolone, acridone, graveoline etc., coumarins like furocoumarin (psoralens), pyranocoumarin and essential oils like 2-nananone, 2- undecyl acetate (Sinshemko et al., 2000). While the pro-oxidant activity of *Ruta* may be due to psoralens, the actual ingredient responsible for the antitumour activity of *Ruta graveolens* extract is not known at present.

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