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## Antiangiogenic Effect of Rutin and its Regulatory Effect on the Production of VEGF, IL-1 $\beta$ and TNF- $\alpha$ in Tumor Associated Macrophages

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**Abstract:** The antiangiogenic activity of rutin was studied using *in vivo* as well as *in vitro* models. *In vivo* angiogenic activity was studied using B16F-10 melanoma cell-induced capillary formation in C57BL/6 mice. Administration of rutin significantly inhibited (43.35%) the number of tumor directed capillaries induced by injecting B16F-10 melanoma cells on the ventral side of C57BL/6 mice. Rutin at non-toxic concentrations (5-25  $\mu\text{g mL}^{-1}$ ) inhibited the vessel sprouting in rat aortic ring assay. Moreover, rutin was able to inhibit the proliferation, migration and capillary-like tube formation of human endothelial cells, the key steps of angiogenesis. Present studies using Tumor Associated Macrophages (TAMs) revealed that rutin could suppress the expression and production of VEGF and IL-1 $\beta$  and stimulate the production of TNF- $\alpha$ . Hence, the observed antiangiogenic activity of rutin is related to the regulation of these cytokines and growth factors by TAMs.

**Key words:** Angiogenesis, cytokines, endothelial cells, rutin, tumor associated macrophages

### INTRODUCTION

Angiogenesis is a complex process, which involves the interactions of several cell types and mediators to establish a specific microenvironment suitable for the formation of new capillaries from pre-existing vessels (Kerbel and Folkman, 2002). The process of forming new blood vessels from an existing vascular bed, normally involves a series of steps, which include endothelial cell activation, breakdown of the basement membrane, migration, proliferation and tube formation of the endothelial cell (Folkman and Shing, 1992). This is a key event that feeds tumor growth and cancer metastasis (Roy *et al.*, 2002). It is widely accepted that angiogenesis is the result of a net balance between the activities exerted by positive and negative regulators. Inflammatory cells, namely monocytes/macrophages, T lymphocytes and monocytes, fully participate in the angiogenic process by secreting pro- and anti-inflammatory cytokines that could control the angiogenic switch (Bergers and Benjamin, 2003; Lingen, 2001). Neoplastic tissues present an inflammatory infiltrate and is characterized by the presence of Tumor Associated Macrophages (TAMs) which have the ability to both positively and negatively regulate tumor growth (Mantovani *et al.*, 1992); tumor cell cytostasis may be induced, but in contrast tumor cell survival may be promoted by release of angiogenic and mitogenic cytokines in the tumor microenvironment such as VEGF

(Barbera-Guillem *et al.*, 2002) and proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  (Kataki *et al.*, 2002; Ghezzi *et al.*, 1991; Naldini *et al.*, 2001; Balkwill and Mantovani, 2001; Cheng and Chen, 2001).

Several polyphenolic compounds are recognized as cancer chemopreventive agents. Flavonoids are especially well known to suppress tumor cell growth via cell-cycle arrest and by induction of apoptosis in several tumor cell lines (Romero *et al.*, 2002; Choi *et al.*, 1998; Davis *et al.*, 1998; Lian *et al.*, 1998; Matsukawa *et al.*, 1993). Moreover, flavonoids inhibit endothelial cell proliferation and angiogenesis *in vitro*, the latter is endothelial cell cultures on collagen gels (Fotsis *et al.*, 1993). Rutin, a member of the flavone family that is present at high levels in many vegetables has been found to possess antitumor (Deschner *et al.*, 1991), anti-inflammatory (Ihme *et al.*, 1996) and antioxidant (Bombardelli and Morrazzoni, 1993; Oomah and Mazza, 1996; Metodiewa *et al.*, 1997) properties. We have reported earlier the antimetastatic activity of rutin in C57BL/6 mouse models (Lata *et al.*, 1995). However the antiangiogenic potential of rutin and its effect on tumor associated macrophages has not been revealed. In this study, we tested the inhibitory effect of rutin on proliferation, migration and tube formation of endothelial cells, the key steps of angiogenesis. Moreover, the effect of rutin on the expression of VEGF, IL-1 $\beta$  and TNF- $\alpha$  on tumor associated macrophages were also evaluated.

## MATERIALS AND METHODS

The present study was carried out in the Department of Immunology, Amala Cancer Research Centre.

**Reagents and chemicals:** Rutin (Fig. 1) was purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of reagent or higher grade.

**ELISA kits:** ELISA kits for mouse IL-1 $\beta$  and TNF- $\alpha$  were purchased from Pierce Endogen (Rockford, USA). Mouse VEGF ELISA kits were purchased from R and D System (Minneapolis, MN).

**Animals:** C57BL/6 (5-6 weeks-old) male mice (20-25 g) were purchased from National Institute of Nutrition, Hyderabad, India. All mice were housed, cared for and used in strict accordance with the rules and regulations of Institutional Animal Ethics Committee, Government of India.

**Cell lines:** Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously (Jaffe *et al.*, 1973). HUVECs were maintained in gelatin-coated 75 cm<sup>2</sup> flasks using M199 (HiMedia, Mumbai, India), supplemented with 20% (v/v) FCS (Biological Industries, Israel), 100 units mL<sup>-1</sup> Penicillin, 100  $\mu$ g mL<sup>-1</sup> Streptomycin, 500 ng mL<sup>-1</sup> basic fibroblast growth factor (bFGF; Pepro Tech Inc. Rocky Hill, NJ) and 100 ng mL<sup>-1</sup> VEGF (Pepro Tech Inc. Rocky Hill, NJ). B16F-10 mouse melanoma cells (National Centre for Cell Sciences, Pune, India) were maintained as a monolayer culture in DMEM (HiMedia, Mumbai, India) supplemented with 10% (v/v) FCS and antibiotics. All the cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Isolation and culture of Tumor Associated Macrophages (TAMs):** Solid tumors (3-4 weeks old) were dissected and mashed aseptically in RPMI-1640 and the cell suspension

was kept undisturbed for 2 h. The supernatant was discarded and the adhered macrophages were scraped out using rubber policeman and cultured in RPMI-1640 supplemented with 10% (v/v) FCS and antibiotics.

**In vivo angiogenesis assay:** Male C57BL/6 mice were divided into two groups (6 animals group<sup>-1</sup>). Angiogenesis was induced by injecting B16F-10 (1 $\times$ 10<sup>6</sup>) cells subcutaneously on the shaved ventral side of the mice. One group was kept as untreated control and the other group was treated with rutin (200  $\mu$ M kg<sup>-1</sup> B.wt.) for five consecutive days. After 9 days, animals were euthanized by cervical dislocation, ventral skin cut removed and the number of tumor directed capillaries per cm<sup>2</sup> around the tumor was counted manually under dissection microscope (10x magnification).

### In vitro angiogenesis

**Rat aortic ring assay:** Rat aorta, collected from 8-10 weeks old male Sprague Dawley rats were cut into 1 mm long cross section and were cultured in collagen coated Twenty-four well tissue culture plates in the presence of conditioned medium (100  $\mu$ L) and escalating concentrations of rutin (5-25  $\mu$ g mL<sup>-1</sup>) of rutin for six days. The number of microvessels sprouted from the aortic ring were counted manually under phase contrast microscope (40x magnification).

**Endothelial cell proliferation assay:** Endothelial cells (5 $\times$ 10<sup>4</sup> cells) were cultured for 24 h in the presence of escalating concentrations of rutin (5-25  $\mu$ g mL<sup>-1</sup>) and the proliferation of endothelial cells were determined by the [<sup>3</sup>H]-thymidine incorporation assay (Lee *et al.*, 1999). The recovered radioactivity was measured using Rack Beta Liquid Scintillation Counter (Wallac 1209, Pharmacia, Sweden) and expressed as counts per minute (cpm).

**Wound migration assay:** Endothelial cells were grown to confluence in 96-well collagen coated culture plates. The endothelial monolayers were wounded using a pipette tip. The media and dislodged cells were aspirated and the wounded cells were incubated in the test medium (M199 + escalating concentrations of rutin). After 48 h incubation, the cells were stained with crystal violet (0.1%) and cells migrated to the wounded area were counted under phase contrast microscope (40x magnification).

**Tube formation assay:** Endothelial cell tube formation was performed by Matrigel Assay (Gupta *et al.*, 2002). Endothelial cells (5 $\times$ 10<sup>4</sup> cells) were cultured for 48 h in Matrigel (Sigma, St. Louis, USA) coated wells in the presence of escalating concentrations of rutin

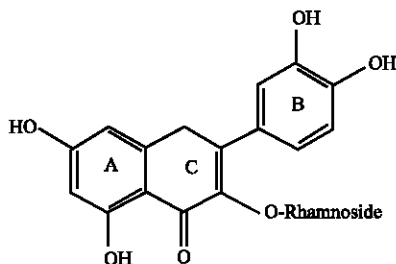


Fig. 1: Chemical structure of rutin

Table 1: Results of PCR analysis using specific primers for VEGF, IL-1 $\beta$ , TNF- $\alpha$  and GAPDH

Gene	Sequence	Product size
IL-1 $\beta$	Forward 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3'	630 bp
	Reverse 5'-CAG GAC AGG TAT AGA TTC TTT CCT TT-3'	
VEGF	Forward 5'-TGC TCA CTT CCA GAA ACA CG-3'	350 bp
	Reverse 5'-GGA ACG GTA AGC CAC TCA CA-3'	
TNF- $\alpha$	Forward 5'-ACT CCC AGA AAA GCA AGC AA-3'	290 bp
	Reverse 5'-TGG AAG ACT CCT CCC AGG TA-3'	
GAPDH	Forward 5'-CGT CCC GTA GAC AAA ATG GT-3'	557 bp
	Reverse 5'-CCT TCC ACA ATG CCA AAG TT-3'	

(5-25  $\mu\text{g mL}^{-1}$ ). The cells were fixed with formaldehyde (5%), stained with Diff Quick (Dade Behring Newark, DE) and photographed under phase contrast microscope (40x magnification).

**Quantification of VEGF, IL- $\beta$  and TNF- $\alpha$  levels:** Tumor associated macrophages ( $5 \times 10^4$  cells) were cultured for 24 h in the presence of escalating concentrations of rutin (5-25  $\mu\text{g mL}^{-1}$ ) and the VEGF, IL-1 $\beta$  and TNF- $\alpha$  levels in the culture supernatant (0.1 mL) were quantified using ELISA kits as per the manufacturer's instructions. V values are expressed in picogram  $\text{mL}^{-1}$ .

**Gene expression studies:** Tumor associated macrophages ( $5 \times 10^4$  cells) were cultured in the presence of escalating concentrations of rutin (5-25  $\mu\text{g mL}^{-1}$ ) for 24 h and the cDNA synthesis was carried out using cDNA<sup>TM</sup> II kit (Ambion) as per the manufacturer's instructions. PCR was performed using specific primers for VEGF, IL-1 $\beta$ , TNF- $\alpha$  and GAPDH (Table 1). The PCR products were electrophoresed on a 1.8% agarose gel and stained with ethidium bromide and photographed under UV light.

**Bioassay for TNF- $\alpha$ :** L929 cells (5000 cells well<sup>-1</sup>) were cultured for 48 h in the presence of TAM culture supernatant (0.1 mL) from the above mentioned experiment. The cells were fixed with formaldehyde (5%), stained with crystal violet (1%) and photographed under phase contrast microscope (40x magnification).

**Statistical analysis:** The data are expressed as mean $\pm$ SD. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Dunnett's test. Unpaired Student t-test was used for statistical analysis, when only two groups are compared. The results were considered statistically significant if the p-values were less than 0.05.

## RESULTS

**Inhibition of angiogenesis by rutin *in vivo*:** Control animals had an average number of 39.9 $\pm$ 1.5 capillaries around the tumor. Rutin treatment showed a significant

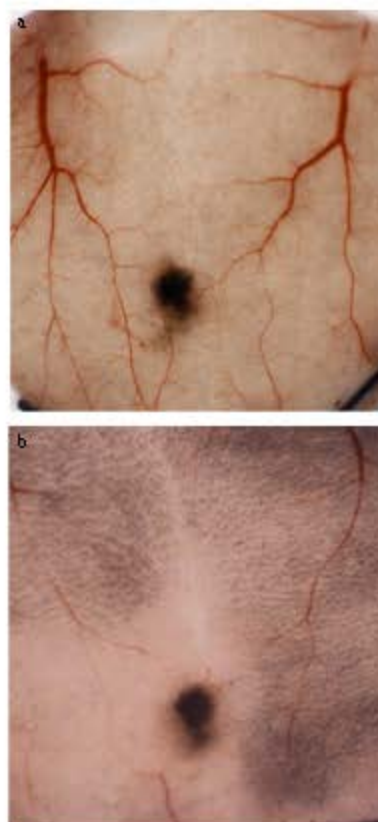


Fig. 2: Inhibition of tumor directed capillaries by rutin *in vivo*. B16F-10 ( $10^6$  cells/mice) were injected s.c. on shaved ventral side of C57BL/6 mice and treated simultaneously with 200  $\mu\text{M kg}^{-1}$  b.wt. for five consecutive days. The tumor directed capillaries were counted at day 9 after implantation of B16F-10 cells. (a) control and (b) Rutin at 200  $\mu\text{M kg}^{-1}$  b.wt.

inhibition (43.35%) in the formation of tumor directed capillaries (Fig 2).

**Effect of rutin on the inhibition of microvessel sprouting:** Incubation of aortic rings with rutin showed a significant and dose dependent inhibition of microvessel

outgrowth from the rat aorta ring induced by the conditioned medium from B16F-10 cells (Fig 3), with 52.8% inhibition in microvessel out growth at a concentration of 25  $\mu\text{g mL}^{-1}$ .

**Effect of rutin on endothelial cell proliferation:** Rutin treatment showed a significant inhibition of endothelial proliferation. Rutin at a concentration of 25  $\mu\text{g mL}^{-1}$  showed 41.02% inhibition in the proliferation of endothelial cells (Fig 4).

**Effect of rutin on endothelial cell migration:** Rutin treatment showed a significant and concentration-dependent inhibition of endothelial cell migration. Rutin at a concentration of 25  $\mu\text{g mL}^{-1}$ , showed 87.5% inhibition

of endothelial cell migration. At 5 and 10  $\mu\text{g mL}^{-1}$ , the percentage inhibition was found to be 32.8 and 42.18%, respectively (Fig 5).

**Effect of rutin on endothelial cell tube formation:** Rutin treatment could significantly inhibit the endothelial cell tube formation in a concentration dependent manner and maximum inhibition was observed at 25  $\mu\text{g mL}^{-1}$  (Fig 6).

**Effect of Rutin on VEGF, IL-1 $\beta$  and TNF- $\alpha$  production and gene expression:** Rutin treatment could significantly inhibit the production of VEGF and IL-1 $\beta$  from TAMs. Rutin at a concentration of 25  $\mu\text{g mL}^{-1}$ , showed 26.81 and 34.21% inhibition in the production of VEGF and IL-1 $\beta$ , respectively. Gene expression studies also showed remarkable inhibition of VEGF and IL-1 $\beta$  mRNA (Fig 7a, b). Rutin treatment could significantly stimulate the TNF- $\alpha$  production and mRNA expression in TAMs (Fig 7c).

**Effect of rutin on TNF- $\alpha$  bioassay:** The culture supernatants from rutin treated TAMs showed maximum cytotoxic effect to TNF- $\alpha$  sensitive L929 cells at a concentration of 25  $\mu\text{g mL}^{-1}$  compared to those treated with 5 and 10  $\mu\text{g mL}^{-1}$  (Fig. 8).

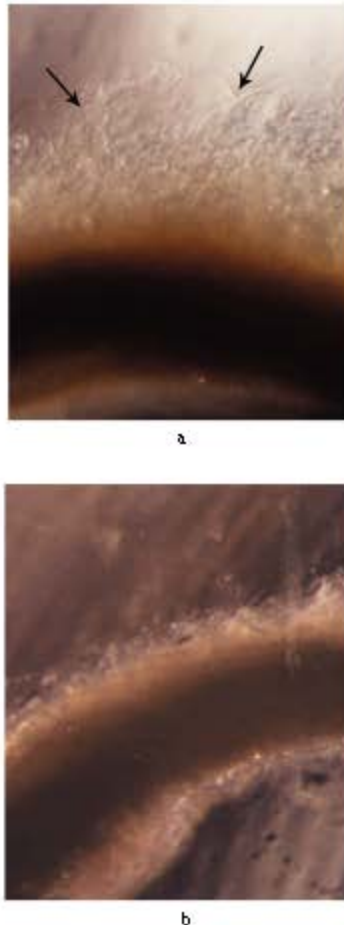


Fig. 3: Inhibition of microvessel sprouting from rat aortic ring by rutin; Microvessel sprouting after 6 days in collagen matrices incubated in (a) conditioned medium from B16-F10 cells and (b) conditioned medium and rutin at 25  $\mu\text{g mL}^{-1}$

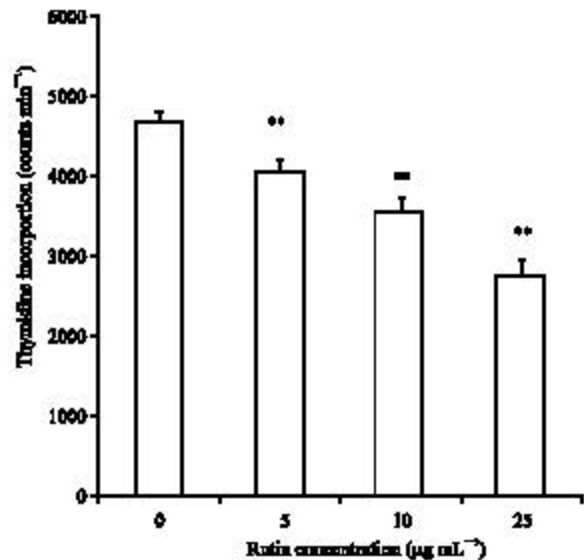


Fig. 4: Inhibition of Human Umbilical Vein Endothelial Cell (HUVEC) proliferation by rutin. HUVEC ( $5 \times 10^4$  cells) were cultured for 24 h in the presence of increasing concentrations of rutin (5-25  $\mu\text{g mL}^{-1}$ ) followed by 18 h incubation after adding [ $^3\text{H}$ ] thymidine (1  $\mu\text{Ci}$ ). Measured radioactivity were expressed as mean counts per minute (cpm)  $\pm$  SD. \*\* $p < 0.001$  rutin treated versus untreated groups; Representative of three separate experiments



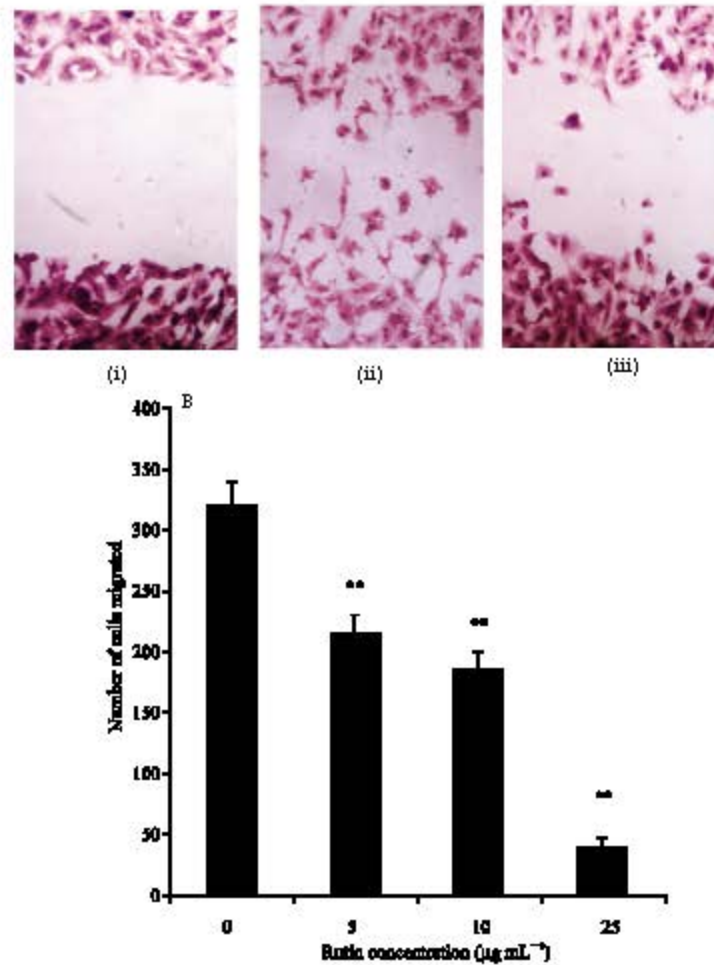


Fig. 5: Crystal violet staining (a) and migration rates (b) of endothelial cells; (a) Wound migration assay was performed on the confluent monolayer of endothelial cells. Increasing concentrations of rutin (5-25 µg mL<sup>-1</sup>) were added to HUVEC monolayer after creating the wound and incubated for 48 h followed by crystal violet (0.1%) staining. (i) 0 h control (ii) 48 h control (iii) 48 h rutin at 25 µg mL<sup>-1</sup> (B) Migration rates of endothelial cells. Values are expressed as mean ± SD (n = 3). The *asterisks* indicate statistic significance (p < 0.001) as a comparison between the treated and respective control groups. Note that rutin produced dose-dependent inhibition of endothelial migration

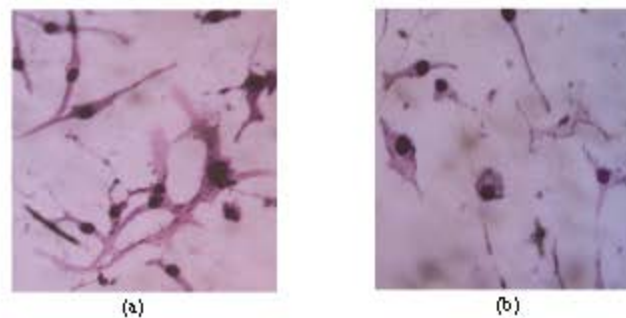


Fig. 6: Diff-Quick staining of endothelial cell tube formation. Endothelial cells ( $5 \times 10^4$ ) were cultured with increasing concentrations of rutin (5-25 µg mL<sup>-1</sup>) and for 48 h followed by Diff-Quick staining (a) 48 h Control and (b) 48 h rutin at 25 µg mL<sup>-1</sup>

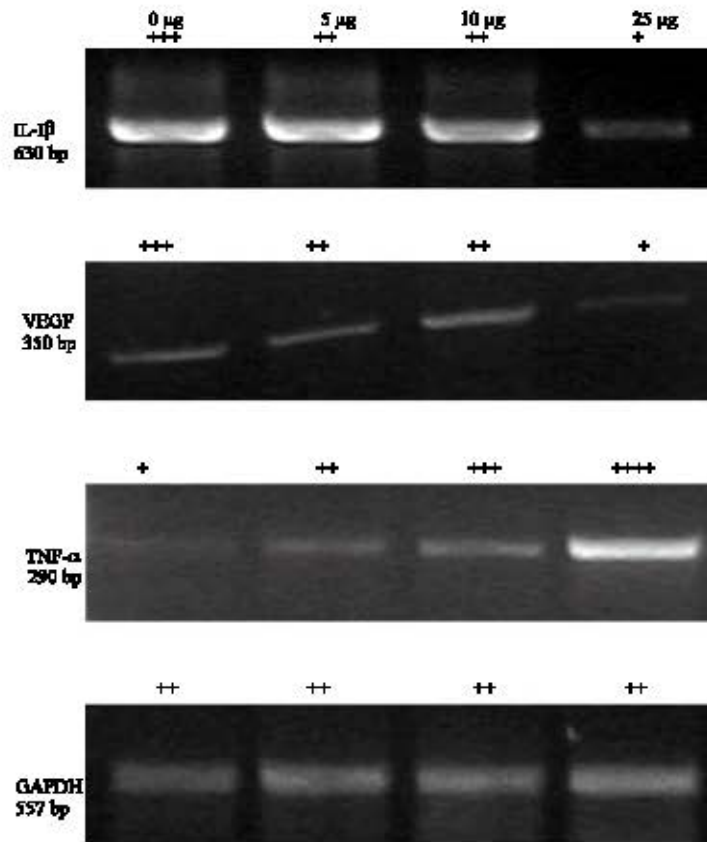


Fig. 7: Effect of Rutin on the expression of IL-1 $\beta$ , VEGF and TNF- $\alpha$  in TAMs. TAMs ( $5 \times 10^4$  cells) were cultured with increasing concentrations of rutin ( $5$ - $25 \mu\text{g mL}^{-1}$ ) for 24 h; cDNA was synthesized followed by amplification using specific primers for IL-1 $\beta$ , VEGF and TNF- $\alpha$ .

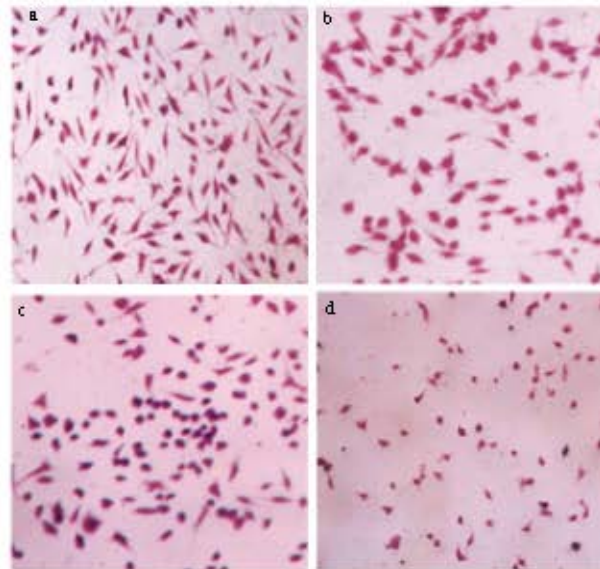


Fig. 8: Effect of Rutin on TNF- $\alpha$  bioassay (a) 48 h control, (b) rutin at  $5 \mu\text{g mL}^{-1}$  (c) rutin at  $10 \mu\text{g mL}^{-1}$  and (d) rutin at  $25 \mu\text{g mL}^{-1}$ .

## DISCUSSION

In the present study we evaluated the inhibitory effect of rutin on the angiogenic process both *in vitro* and *in vivo*. We for the first time showed that rutin could alter the VEGF, IL-1 $\beta$  and TNF- $\alpha$  production in tumor associated macrophages. It has been proved conclusively that angiogenesis is essential for a tumor to become large and malignant; hence inhibition of the process of angiogenesis either by inhibiting endothelial cells or by suppression of angiogenic growth factors should provide a novel therapeutic window for cancer therapy. Here in, we focused on studying the potential effect of rutin on the process of angiogenesis itself using vascular endothelial cells as a model. We demonstrated that rutin could inhibit tube formation of HUVECs using *in vitro* matrigel assay. Matrigel is a matrix of mouse basement membrane neoplasm, which represents a complex mixture of basement membrane proteins including type IV collagen, entactin/nitrogen, proteoheparan sulfate and other growth factors. Matrigel can induce endothelial cells to differentiate as shown by morphological changes and is widely accepted model to study biochemical changes associated with angiogenesis (Ponce *et al.*, 2001). The inhibition of tube formation, as observed in our study after rutin treatment might be due to the reduced proliferation of endothelial cells. The concentrations of rutin used in the present studies did not interfere with the cell viability of endothelial cells. The decreased number of tumor directed capillaries in rutin treated animals also indicates its potential to selectively inhibit the tumor specific capillary sprouting. This inhibition of capillary formation might be one of the reasons for the antimetastatic effect of rutin (Lata *et al.*, 1995).

A prime regulator for angiogenesis is believed to be Vascular Endothelial Growth Factor (VEGF) and low oxygen tension dramatically induces the expression of this major angiogenic factor in tumor associated macrophages (Schoppmann *et al.*, 2002; Forsythe *et al.*, 1996; Schioppa *et al.*, 2003). VEGF expression is closely associated with increased intratumoral microvessel density in breast cancer patients (Toi *et al.*, 1996). Rutin treatment showed significant inhibition of microvessel sprouting from aortic ring induced by VEGF rich conditioned medium. Quantification of growth factor VEGF by ELISA in our studies indicated a decreased amount of VEGF in rutin treated TAM culture supernatant. This may be due to the down regulatory action of rutin on the VEGF mRNA expression as there was only low level expression of VEGF mRNA when TAMs were treated with rutin.

During tumor development, the cross-talk between tumor associated macrophages and surrounding cells is crucial. With regards to the cross-talk between inflammatory cells and endothelial cells present in the tumor microenvironment, IL-1 $\beta$  stimulates the proliferation of endothelial cells, adhesion molecule expression and production of cytokines and inflammatory molecules *in vitro*; suggesting that IL-1 related molecules may be deeply involved in the control of angiogenesis process (Dianarello, 1996, 1998). IL-1 $\beta$  is a pleiotropic cytokine that induces immunosuppression under different experimental conditions (Dianarello, 1996). The relevance of microenvironmental IL-1 in angiogenesis and in the invasiveness of different tumor cells has been recently demonstrated using IL-1 $\beta$  knockout (KO) mice (Voronov *et al.*, 2003). IL-1 $\beta$  upregulates HIF-1 $\alpha$  in a human lung cancer cell line (A549) activating NF- $\kappa$ B/Cox-2 pathway (Jung *et al.*, 2003). Present studies revealed that rutin could inhibit the expression of IL-1 $\beta$  in TAMs. The inhibition of IL-1 $\beta$  expression by rutin as observed in our results also might have occurred through similar mechanisms. Further studies in this area are under progress to fully elucidate the mechanism of action of rutin on the regulation of IL-1 $\beta$  in TAMs.

In inflammation and angiogenesis, TNF- $\alpha$  plays a dual role: it controls both tissue destruction and recovery and these roles are determined by the context in which this cytokine acts (Fajardo *et al.*, 1992). With regards to cancer development and progression, high doses of TNF- $\alpha$  induce death of tumor cells (Lejeune *et al.*, 1998). However, low concentrations of TNF- $\alpha$  promote tumor growth and spread (Foa *et al.*, 1990). Thus TNF- $\alpha$  might stimulate tumor development by promoting vessel growth and participate in tumor and endothelium destruction by direct cytotoxicity. Present studies reveal that rutin treatment could stimulate the production of TNF- $\alpha$  in TAMs and these levels could induce cytotoxicity to tumor cells as evidenced by TNF- $\alpha$  sensitive L929 bioassay.

In conclusion, the present study demonstrates the antiangiogenic potential of rutin and its regulatory effect on the production of VEGF, IL-1 $\beta$  and TNF- $\alpha$  in TAMs. Since macrophages present within the solid tumor contribute to the initiation of angiogenesis, the present study is of significant relevance of developing rutin as potential therapeutic target against TAMs in controlling angiogenesis.

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