RUTIN INHIBITS NITRIC OXIDE AND TUMOR NECROSIS FACTOR-α PRODUCTION IN LIPOPOLYSACCHARIDE AND CONCANAVALIN-A STIMULATED MACROPHAGES

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SUMMARY

The effect of rutin, a flavonoid present in onions, apples, tea and red wine, on the production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) was analyzed using *in vitro* as well as *in vivo* systems. The level of nitrite in lipopolysaccharide (LPS) stimulated BALB/c mice (88.21 μM) was significantly reduced in rutin treated animals (16.92 μM). The nitrite level in concanavalin-A (Con-A) treated control animals (77.15 μM) was also significantly reduced to 11.03 μM when the animals were pretreated with rutin. The drastically elevated levels of TNF-α in LPS stimulated animals (686.8 pg/ml) was lowered by pretreatment with rutin (182.4 pg/ml). Rutin also inhibited Con-A induced TNF-α production. Rutin inhibited nitrite production by activated macrophages *in vitro* (74.75 μM) to the normal level (16.13 μM) at a concentration of 5 μg/ml. *In vitro* L929 bioassay also showed inhibition of TNF-α production by rutin treatment.

KEY WORDS

rutin, nitric oxide, tumor necrosis factor, lipopolysaccharide, concanavalin-A, bioassay

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INTRODUCTION

Macrophages are known to play an important role in host defense mechanisms. Among a variety of mediators released by activated macrophages /1,2/, nitric oxide (NO) has been identified as a potent molecule that may exert regulatory or cytotoxic effect, depending on the concentration acting on the target cell /3,4/. In mammals, NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The products of L-arginine oxidation by NOS are L-citrulline and NO. NO has a very short half life, is lipid soluble, reacts easily with several enzyme systems, and is produced by a wide range of cells /5-8/. NO acts as an important mediator of tissue damage in inflammatory diseases; elevated NO levels are thought to play a central role in tissue damage observed during septic shock /9,10/. The biochemical mechanisms of NO-induced cytotoxicity are not completely understood. There is an NO-dependent inhibition of key enzymes in the respiratory cycle and in the synthesis of DNA in target cells, in some cases involving reaction with iron-sulfur centers on these enzymes. Studies suggest that NO may inhibit cytochrome *c* oxidase, the terminal electron transport protein in mitochondria /11/. This blockade could result in the local formation of $O_2^{\bullet}$ radicals in the respiratory chain and the destruction of iron-sulfur centers by $O_2^{\bullet}$ itself or via the formation of the potentially damaging species peroxynitrite ($ONOO^{-}$) /12/. Other actions of NO independent of the respiratory chain could also contribute to its cytotoxicity, because after oxidation, NO may cause nitrosative deamination of nucleic acids with consequent DNA stand breaks and mutagenicity /13/. High concentrations of NO have deleterious effects, so it is necessary that the production of NO be tightly regulated /14/.

Tumor necrosis factor-α (TNF-α), also known as cachectin, is a protein product of activated macrophages that plays a central role in integrating and amplifying the host response to infection and malignancy /15,16/. Through its interaction with cells such as macrophages, fibroblasts and endothelial cells, TNF promotes the immune response, local inflammatory processes, and wound repair /17-19/. TNF-α is also known to elicit cytotoxicity and mediate the wasting (cachexia) that accompanies disease states such as AIDS /20,21/.

The binding of TNF with its cell surface receptors has been shown to activate complex signal transduction pathways, including activation

of protein kinase C, generation of reactive oxygen intermediates, activation of NF-κB, and induction or downregulation of growth regulatory genes, such as bcl-2, c-myc and p53 /22,23/. The exact mechanism whereby these events mediate the TNF-α induced cytotoxicity in mammalian cells is not well established although oxidation damage that leads to cell apoptosis or programmed cell death is suggested by many investigators /24/.

Earlier studies have shown that functions of activated macrophages, such as killing tumor cells, release of cytokines, and generation of oxygen radicals, can be regulated by flavonoids /25-27/. Flavonoids represent the most common and widely distributed group of plant phenolics /28/ and are abundant in foods. Rutin is one of the flavonoids abundantly consumed in foods /29/ and it is present in onions, apples, tea and red wine /30/. Rutin exhibits multiple pharmacological activities, including antibacterial, antitumor, anti-inflammatory, antidiabetic, myocardial protective, vasodilatator and hepatoprotective activities /31,32/. The present study was designed to investigate the effect of rutin on the inhibition of NO and TNF-α production in both *in vitro* and *in vivo* systems.

MATERIALS AND METHODS

Animals

Male BALB/c mice (20-25 g) obtained from the Animal House, Amala Cancer Research Centre, were used in this study. The animals were fed a standard pellet diet (Sai Durga Feeds, Bangalore, India) and water was freely available. They were maintained in a controlled environment (12:12 h light/dark cycle) and temperature (30 ± 2°C). The experimental protocol was approved by the Animal Ethics Committee, Government of India.

Cells

L929 lung fibroblast cell line was obtained from the National Centre for Cell Sciences, Pune, India. The cells were maintained in MEM supplemented with 10% goat serum and antibiotics.

Chemicals

MEM and RPMI-1640 were purchased from Hi-Media Lab, Mumbai, India. LPS (*Eschericia coli* serotype 026:BG) was purchased from Difco Laboratories, Detroit, MI, USA. Rutin and concanavalin-A (Con-A) were purchased from Sigma Chemical Co., USA. Rutin was suspended in 0.1% gum acacia for *in vivo* studies. For *in vitro* studies, rutin was suspended in DMSO (stock) and further diluted with the medium. All chemicals used in the present study were of analytical grade.

Isolation of peritoneal macrophages

Sodium caseinate-elicited peritoneal exudate cells were obtained from 5 week-old BALB/c mice following intraperitoneal injection of 0.2 ml sodium caseinate (5 g/100 ml) and lavage of the peritoneal cavity with 5 ml sterile saline 5 days later. The cells were washed twice with PBS (pH 7.4) and resuspended in RPMI-1640 (containing 10% FCS, 2 mM L-glutamine, and 100 μg/ml streptomycin and penicillin). Peritoneal exudate cells were seeded at densities of 2×10^5 cells/well on 96-well titer plates (Tarsons) and the macrophages were allowed to adhere for 2 h in 5% CO_2 humidified atmosphere. The non-adherent macrophages were incubated in complete culture medium (RPMI-1640 supplemented with 10% FCS and antibiotics). Macrophages were cultured with or without LPS (5 μg/ml) in the presence and absence of rutin (1, 5, 10 and 25 μg/ml) for 24 h at 37°C in 5% CO_2. After 24 h the plates were centrifuged and the supernatant was used for the estimation of NO and TNF-α.

Estimation of nitrite levels

Nitrite, an indicator of NO synthesis, was measured in the supernatant by the Griess reaction /33/. In short, 100 μl samples were incubated with an equal amount of Griess reagent, which consists of one part 0.1% *N*-(1-naphthyl)-ethylene diamine dihydrochloride in distilled water and one part 1% sulfanilamide in 5% concentrated H_3PO_4, and incubated for 10 min at room temperature. Absorbance at 540 nm was determined using a microwell plate reader (Awareness Technology Inc., USA). The amount of nitrate was calculated from a $NaNO_2$ standard.

Estimation of TNF-α *in vitro*

TNF-α levels in the samples were determined using ELISA (PIERCE Endogen Inc., Rockford, USA). In this method, the microwell plates were coated with antibody to TNF-α. TNF-α present in the test or standard sample binds to antibodies adsorbed to the microwells. A biotin-conjugated TNF-α antibody was added to detect TNF-α captured by the first coated antibody. Following incubation, the unbound biotin-conjugated anti-TNF-α was removed by washing. Streptavidin-horseradish peroxidase (HRP) was added to bind the biotin-conjugated anti-TNF-α. Following incubation, unbound streptavidin-HRP was removed during a subsequent washing step. Substrate solution reactive with HRP was finally added to the wells. A colored product was formed in proportion to the amount of TNF-α present in the sample. The reaction was terminated by adding stop solution, and absorbance was measured at 450 nm. The concentration of TNF-α was calculated from a standard curve.

Bioassay for TNF-α production

L929 cells were seeded in a 96-well titer plate at a density of 5,000 cells/well. Macrophages (2 x 10^5 cells/well) were cultured in the presence and absence of rutin (1, 5, 10 and 25 μg/ml) for 24 h at 37°C in 5% CO_2. After 24 h the plates were centrifuged and the macrophage-containing supernatant was added to the wells containing target L929 cells in triplicate. The plates were incubated at 37°C in 5% CO_2 atmosphere for 48 h. After incubation cells were fixed and stained with crystal violet, and cellular cytotoxicity was assessed morphologically.

Determination of the effect of rutin on the production of NO and TNF-α by stimulated macrophages *in vivo*

BALB/c mice weighing 20-25 g were separated into five groups (6 animals/group). Group I animals were treated with LPS (250 μg in 0.5 ml PBS) alone, group II was treated with LPS (250 μg in 0.5 ml PBS) and rutin (200 μM/kg body weight/animal/dose) intraperitoneally. Group III was treated with Con-A (100 μg in 0.5 ml PBS) alone, and group IV treated with Con-A (100 μg in 0.5 ml PBS) and rutin (200 μM/kg body weight/animal/dose) intraperitoneally. Group V was kept

as untreated control. Rutin treatment was started four days prior to the LPS and Con-A treatments. Six hours after LPS or Con-A treatment blood was collected from the tail vein and serum was separated.

Nitrite level in the serum was estimated by a microplate assay method using the Griess reagent as explained above /33/. The amount of nitrite present in the serum was calculated from an $NaNO_2$ standard curve. Serum TNF-α level was estimated using ELISA as explained above.

Statistical analysis

All results are expressed as means ± standard deviation. Student's t-test was used to determine the level of correlation between the groups. Results with $p < 0.05$ were considered statistically significant.

RESULTS

Effect of rutin on the release of nitrite by activated macrophages

The effect of rutin on the release of nitrite by activated macrophages is shown in Figure 1. Macrophage cultures stimulated with LPS (5 μg/ml) for 24 h produced elevated levels of nitrite (74.75 μM) compared to normal unstimulated macrophages (16.98 μM). Rutin inhibited NO production in a dose dependent manner. Rutin at concentrations of 1, 5, 10 and 25 μg/ml reduced the nitrite production to 22.82, 16.13, 13.66, and 10.75 μM, respectively, in LPS stimulated macrophages.

Effect of rutin on the release of TNF-α by activated macrophages *in vitro*

TNF-α released by activated macrophages after treatment with rutin is shown in Figure 2. The low level of TNF-α released by normal unstimulated macrophages (38.60 pg/ml) was found to be drastically enhanced on treatment with LPS (766.4 pg/ml). Administration of rutin at 1, 5, 10, and 25 μg/ml significantly reduced TNF-α production in LPS stimulated macrophages to 564.86, 324.32, 256.66, and 184.52 pg/ml, respectively.

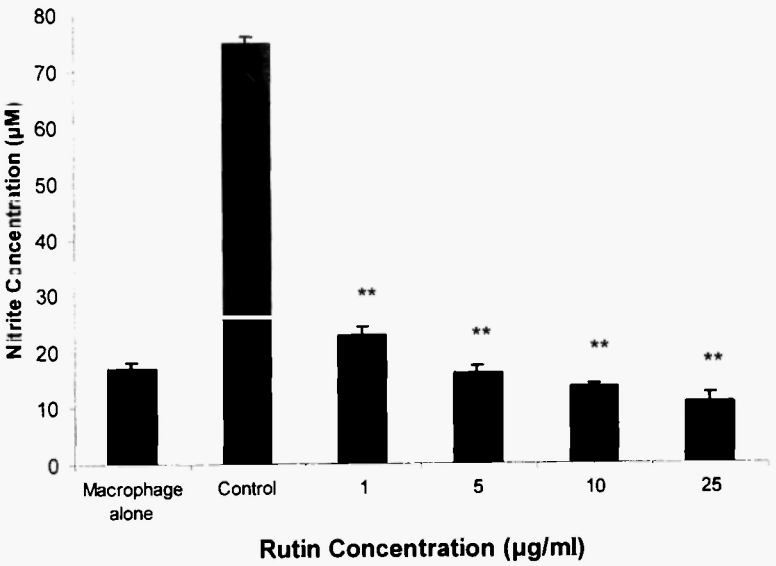

Fig. 1: Effect of rutin on nitrite production by lipopolysaccharide (LPS) stimulated macrophages. Peritoneal macrophages were harvested and 2 x 10^5 cells were plated in 96-well titer plates. The cells were cultured in the presence and absence of LPS as well as rutin. Culture supernatant was collected and nitrite concentration was estimated. Values are expressed as mean ± SD. ** $p < 0.01$, statistically significant compared to control.

Effect of rutin on the release of TNF-α by activated macrophages (bioassay)

Culture supernatant collected from LPS activated macrophages produced 100% cytotoxicity to the TNF-α sensitive L929 cell line (Fig. 3a). Culture supernatant collected from the rutin treated (25 µg/ml) LPS stimulated macrophages produced less cytotoxicity to the L929 cells (Fig. 3b), and cell growth was normal with untreated macrophage supernatant (Fig. 3c). Cytotoxicity of the L929 cells by TNF-α was highly reduced under rutin treatment, indicating that rutin can inhibit the production of TNF-α by LPS stimulated macrophages.

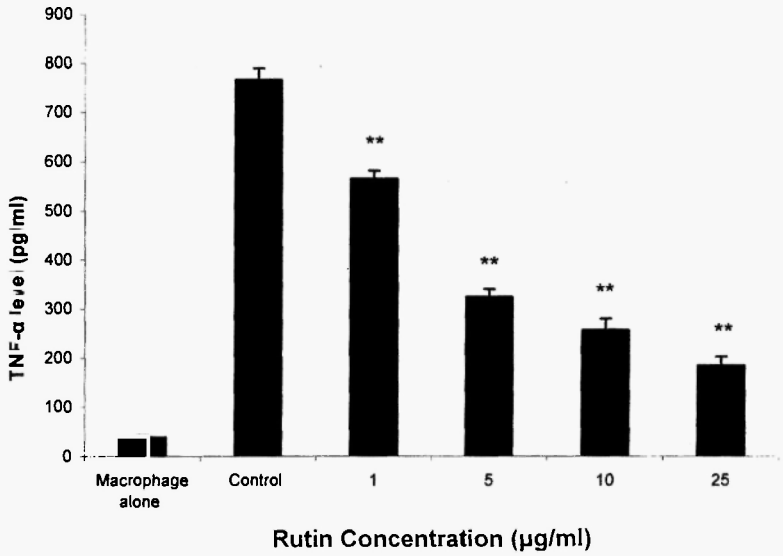

Fig. 2: Effect of rutin on TNF-α production by lipopolysaccharide (LPS) stimulated macrophages. Peritoneal macrophages were harvested and 2 x 10^5 cells were plated in 96-well titer plates. The cells were cultured in the presence and absence of LPS as well as rutin. Culture supernatant was collected and TNF-α level was estimated using ELISA. Values are expressed as mean ± SD. ** $p < 0.01$ statistically significant compared to control.

Effect of rutin on the production of serum nitrite in LPS and Con-A stimulated animals

The elevated level of serum nitrite after LPS stimulation (88.21 μM) was significantly reduced to 16.92 μM after rutin treatment, which was similar to that of normal animals (21.26 μM) (Fig. 4). Similar results were also observed after rutin administration in Con-A treated animals. The elevated level of nitrate in Con-A treated animals (77.15 μM) was significantly reduced to 11.03 μM after treatment with rutin.

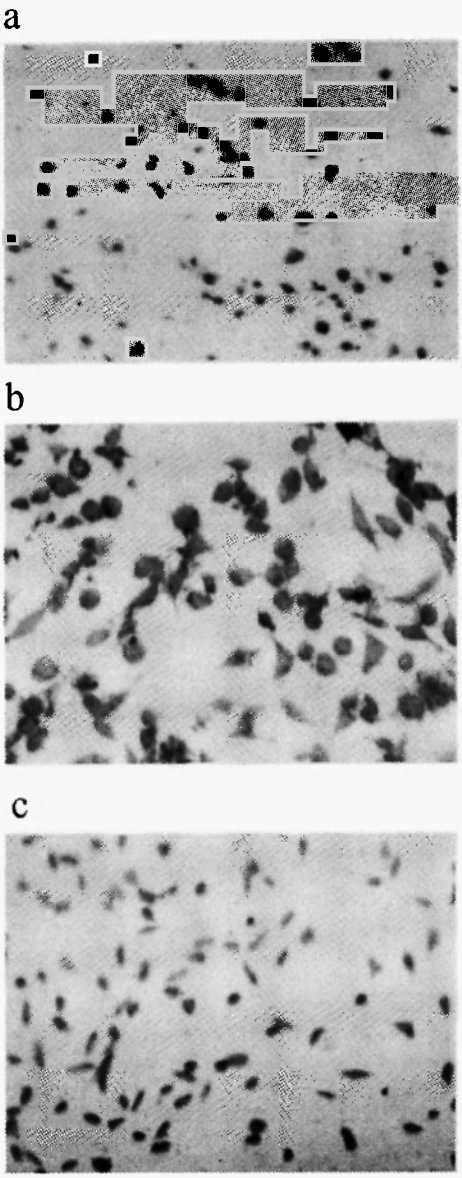

Fig. 3: Effect of rutin on the release of TNF-α by activated macrophages (bio-assay). L929 cells incubated with supernatant from a) lipopolysaccharide (LPS) stimulated macrophage culture; b) LPS stimulated macrophages in the presence of rutin (200 μM); and c) untreated normal macrophage culture.

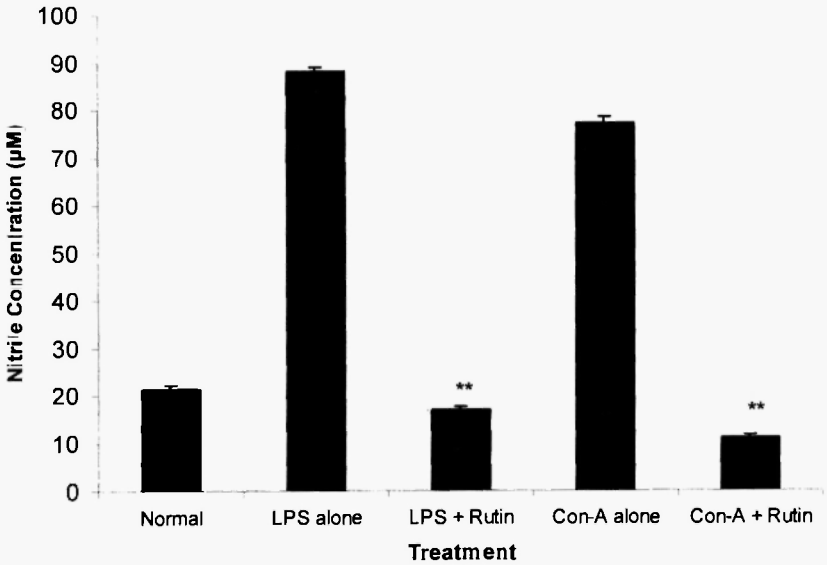

Fig. 4: Effect of rutin on serum nitrite levels of lipopolysaccharide (LPS) and concanavalin-A (Con-A) stimulated animals. BALB/c mice were treated with rutin (200 μM/kg BW/dose) for 4 days prior to LPS or Con-A stimulation. After 6 h of LPS or Con-A stimulation blood was collected and serum used for nitrite estimation. Values are expressed as mean ± SD. ** $p < 0.01$ statistically significant compared to control.

Effect of rutin on the production of serum TNF-α in LPS and Con-A stimulated animals

The effect of rutin on serum TNF-α is shown in Figure 5. Treatment with LPS drastically enhanced serum TNF-α to 686.8 pg/ml. Rutin treatment reduced the level to 182.4 pg/ml. Similarly, in rutin treated Con-A stimulated animals, a significant decrease in the amount of TNF-α (96.6 pg/ml) was noted.

DISCUSSION

Chronic increases in oxidative stress and NO production in cells are key biochemical events that have been linked to cancer and

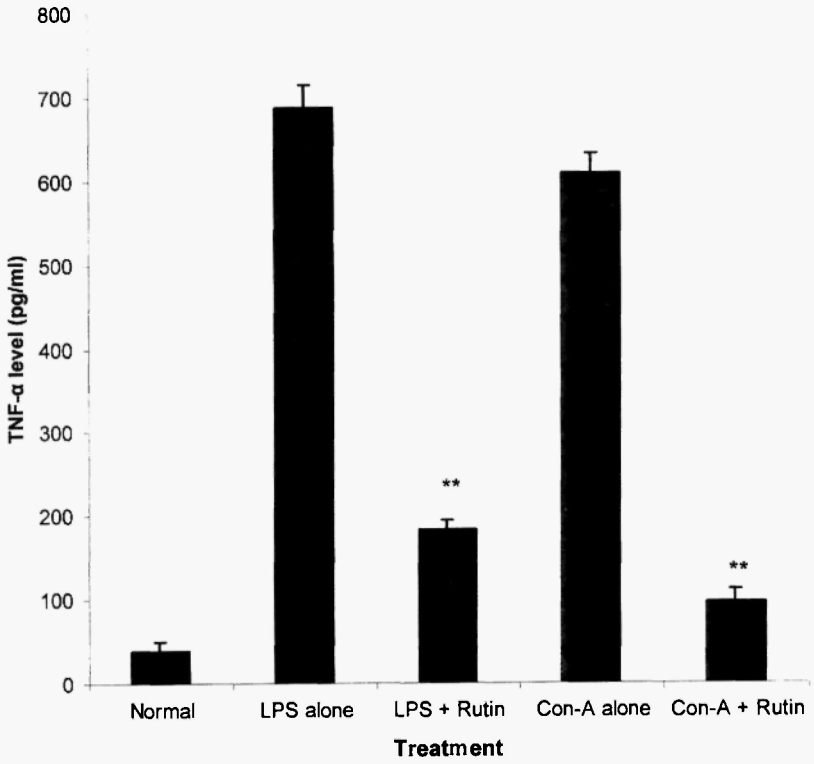

Fig. 5: Effect of rutin on serum TNF-α levels of lipopolysaccharide (LPS) and concanavalin-A (Con-A) stimulated animals. BALB/c mice were treated with rutin (200 μM/kg BW/dose) for 4 days prior to LPS or Con-A stimulation. After 6 h of LPS or Con-A stimulation blood was collected and serum used for TNF-α estimation. Values are expressed as mean ± SD. ** $p < 0.01$ statistically significant compared to control.

inflammation. Overproduction of reactive oxygen species (ROS) can lead to a wide range of toxic, oxidative reactions, resulting in enormous physiological and pathological damage /34/. Prolonged NO generation has attracted attention because of its relevance in carcinogenesis. It also plays important roles in inflammatory responses /35/. Several studies have shown that antioxidant agents are able to decrease the occurrence of inflammation and cancer by decreasing oxidative stress, prostaglandin secretion and NO production in cells via the NF-κB pathway /36/.

Injection of LPS or Con-A in mice induces a significant increase in serum nitrite and nitrate (NO_x) levels, indicating elevated production of NO /37/. Inducible NO synthase (iNOS) is the enzyme responsible for enhanced NO production during inflammation /38/. Increased NO synthesis during experimental endotoxemia has been shown to have beneficial effects; however, high concentrations of NO can also have deleterious effects /38-42/. Therefore, it is necessary that the production of NO be tightly regulated. TNF-α, a proinflammatory cytokine released during endotoxemia, is likely to be involved in the regulation of NO production since *in vitro* data show that TNF-α plays an important role in the upregulation of NO synthesis /43-48/. TNF-α is a peptide mediator released by monocytes and macrophages in response to various stimuli including bacterial LPS /49/. TNF-α has been shown to enhance the oxidative metabolism of human mononuclear phagocytes /50/. This results in the release of reactive oxygen intermediates, including superoxide and hydrogen peroxide, which may cause cytostasis or cytotoxicity /51/. TNF-α plays an important role in the regulation of inflammatory processes and has been implicated in the pathogenesis of inflammatory conditions such as rheumatoid arthritis and Crohn's disease /52/. Inhibiting TNF-α with various biologicals, such as infliximab (a mAB) and etanercept (an STNF-R-Fc fusion protein) has been shown to be very effective in the treatment of these conditions /53/.

Our data strongly suggest that rutin could inhibit the production of nitrite and TNF-α both *in vitro* and *in vivo*. The cellular mechanisms of the effect are not clear, but may be related to the known biological effects of rutin, such as antioxidant properties and inhibition of cellular enzymes involved in signal transduction /54,55/. The primary mechanism of anti-inflammatory actions of several plant extracts and their role in immunomodulation is through inhibition of TNF-α production and scavenging of free radicals /56/. Thus the ability of rutin to inhibit NO production and TNF-α may have a therapeutic implication. Studies as to whether rutin acts as a direct inhibitor of NOS or acts on a control system are in progress, and will be reported separately.

ACKNOWLEDGEMENT

The authors thank Dr. Ramadasan Kuttan, Director, Amala Cancer Research Centre, for his consistent support during the period of study.

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