Indian Journal of Biochemistry & Biophysics
Vol. 57, February 2020, pp. 58-63

# Immunomodulatory effect of rutin, catechin, and hesperidin on macrophage function

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*Received 19 June 2019*; *revised 15 October 2019*

The inflammation was observed by a sequence of comprehensive, correlated prevalence which causes enrollment of macrophages along with by removal of foreign particles and instigation of tissue repair. Though, massive release of chemokine and cytokines from macrophages can cause the progression of auto-inflammation and auto-immune diseases. The control overproduction of these mediators without damaging macrophages seems to be a novel strategy to control inflammation. Plants have been an integral part of the health-care system since ancient times. Many modern-day pharmaceuticals find their origin from phytochemicals. Rutin, catechin, and hesperidin are well-recognized for analgesic, anti-inflammatory and antiarthritic effects. Scientific reports rationalize their protective effect on humoral and cell-mediated immunity. The present study is focused to determine the immunomodulatory effect of rutin, catechin, and hesperidin on macrophage function. Rat peritoneal macrophages were harvested and cultured in the presence of lipopolysaccharides (LPS) and hesperidin. MTT assay was performed to determine macrophage viability. The levels of TNF-α, IL-1β, IL-6 were determined by ELISA kits whereas NO levels were determined by Griess method. The results of the present study revealed a decrease in levels of TNF-α, IL-1β, IL-6 and NO in LPS treated groups. In conclusion, LPS activated the macrophage by promoting the production of immune mediators, whereas rutin, catechin, and hesperidin treatment showed anti-inflammatory activities by suppressing the cytokine production in rat macrophages.

**Keywords**: Flavonoids, Interleukin 1β, Interleukin 6, Modulation, Nitric oxide, Tumor necrosis factor-α

Macrophages are the important class of immune cells that are known to play a key role in acquired and innate immunity [1,2]. They are the prime cells that contribute to initiating an immune response. The key functions of macrophages include destruction/ phagocytosis of foreign particles along with the production of cytokines, COX-2 and NO. Macrophages are involved in the mediation of diseases like hepatocellular damage, cardiac damage, rheumatoid arthritis[3], ulcerative colitis, and DNA damage[4]. Such dysregulation causes inflammatory injury in various organs leading to increased morbidity and poor quality of life[5]. Chemotherapeutic agents are used to suppressing such states; however; there are various adverse effects associated with these therapies[6,7]. This has led to rising attention in recognizing and characterizing natural compounds from plants with immunomodulatory effects with low toxicity.

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Rutin, a bioflavonoid is ubiquitously found in all plants. Apples and tea leaves carry an abundant amount of rutin[8,9]. Rutin possesses therapeutic as well as nutraceutical potential[10]. Major therapeutic effects of rutin include analgesic, anti-inflammatory, organ protection, anti-microbial and anticancer effects[11]. Recently a report revealed a protective effect of rutin on cellular and humoral immunity in rat model[12]. In this study, rutin administration caused a significant increase in antibody titer and total antibody levels. There was also an increased clearance of carbon particles from the blood. Based on the above observation, the present study was aimed to determine the immunomodulatory effect of rutin on macrophage function. Catechin is a plant-derived secondary metabolite widely found in nature belonging to flavonols family. The word 'catechin' is originated from catechu, obtained from the boiled extract of *Mimosa catechu*[13]. Sources of catechin include green tea, Korean tea, black tea, coconuts, onion, grape seeds[14] and much more. Catechin demonstrated 'various pharmacological effects' on the biological

system out of which analgesic[15], anti-inflammatory[16] and antarthritic[17] effects are noteworthy. In a study, catechin administration caused enhancement in delayed-type hypersensitivity and aided in restoration in the functioning of leucocytes in cyclophosphamide-treated rats along with increased clearance of carbon particles[18]. Hesperidin, a flavan-on, is a by-product of Citrus cultivation. It is known to prevent capillary permeability and fragility[19]. In a study, hesperidin supplementation aided in lowering edema/swelling and prevents fluid accumulation[20]. Hesperidin has also been studied for its beneficial effects on certain organ systems. The present work aimed to determine the immunomodulatory effects of rutin, catechin, and hesperidin on the rat macrophages.

## Materials and Methods

### Chemicals

Hesperidin was purchased from Central Drug House, India. The thioglycollate medium was purchased from Central Drug House, India. Lipopolysaccharide (LPS) from *E. coli* was obtained from Santa Cruz Biotechnology, USA. ELISA kits for TNF-α, IL-1β, and IL-6 were purchased from Krishgen Biosystems, USA. N-alpha-naphthyl-ethylenediamine was purchased from Finar, India. Fetal bovine serum and RPMI were purchased from Invitrogen, India. All the other chemicals used were of analytical grade.

### Animals

Wistar rats (180-200 g) of either sex were housed in polypropylene cages, maintained under standardized conditions (12 h light/dark cycles, 28±2°) were used in the study. Animals were provided with standard pellet food and had free access to drinking water. All the animal study protocols were duly approved by the Institutional Animal Ethics Committee.

### Isolation of rat peritoneal-exudate macrophages

For induction of inflammatory responses, rats were injected intraperitoneally with 1 mL of 3% thioglycollate broth, and peritoneal exudate was extracted 5 days later. Rat resident and inflammatory peritoneal macrophages were obtained from rats. The abdomen of the rat was soaked with 70% ethanol for disinfection, a midline incision was then made with scissors, and the abdominal skin retracted. 30 mL Hanks' balanced salt solution (HBSS) was then injected into the peritoneal cavity using a syringe with a 19-G needle. After the gentle abdominal massage, about 30 mL of peritoneal fluid was extracted using the same syringe and transferred to 50 mL sterile polypropylene tubes on ice. A 20 µL aliquot was then extracted for cell counting in a hemocytometer. The remaining cells were washed once by centrifugation at 400 rpm for 10 min at 4°C and resuspended to a concentration of $10^6$ cells/mL. The number of viable cells was estimated by the trypan blue exclusion test: trypan blue (0.4% in PBS) was added to wells and incubated for 3 min at room temperature (25°C), after which the number of unstained (viable) and stained (nonviable) were counted. Aliquots of 100 µL of the cell suspension were added to the wells of 96-well microculture plates (Corning, USA) or placed on microscope slides, and left for 90 min in a humidified incubator (37°C, 5% $CO_2$) to allow adhesion. Nonadherent cells were then removed by gently washing with HBSS[21].

### Determination of the inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6)

The concentrations of inflammatory cytokines and chemokines in cell culture medium in the presence and absence of lipopolysaccharides (from *E. coli*) were determined by using Krishgen ELISA based kits as per manufacturer's instructions.

### Determination of Nitric oxide

After stimulation of macrophages ($1 \times 10^5$ cells) with LPS (1 µg/mL) for 24 h in the presence of various concentrations of each drug, nitrite levels in the conditioned medium were determined using Griess reagent[22]. Macrophage cells ($1 \times 10^5$ cells/mL) were dispensed into a 6-well plate for 24 h. Cells were stimulated with LPS (1 µg/mL) and treated with various concentrations of hesperidin (5, 10 and 25 µg/mL) for 24 h. After incubation, 50 µL of culture supernatants were mixed with an equal volume of Griess reagent in 96-well plate and incubated at 25°C for 10 min. The absorbance at 570 nm was measured on a microplate reader. Nitrite concentrations in culture supernatants were measured to assess NO production in macrophages. $NaNO_2$ was used as a standard to calculated nitrite concentrations[23].

### Statistical analysis

Data is expressed as Mean ± SD and statistical analysis was carried out employing the one-way ANOVA followed by Bonferroni multiple comparison test. $P$ values $< 0.05$ are being taken as statistically significant.

## Results

### Cell viability

MTT assay is extensively used to determine the activity of mitochondrial enzyme succinate dehydrogenase. This protocol is generally utilized to determine the effect of chemicals from the natural and synthetic origin over the biological system. In the present work, the MTT assay was used to access the effect of hesperidin on the viability of rat macrophage. Hesperidin supplementation caused no harmful effect over the cultured rat peritoneal cells. There was a cell proliferation by 98 % for rutin, 98 % for catechin and 99 % for hesperidin.

### Effect on cytokine levels

There was a noteworthy increment in the levels of cytokines due to treatment with lipopolysaccharides. Conversely, the treatment with rutin, catechin, and hesperidin caused a significant reduction in cytokine levels. Briefly, there was a decrease in TNF-α levels in the cells treated with hesperidin as compared to control (LPS only) in a concentration-dependent manner. There was a significant decrease in the level of this cytokine at 100 µM ($P$ <0.001) (Fig. 1A). Hesperidin followed by rutin and catechin (1 and 10 µM) caused a significant ($P$ < 0.001) decrease in IL-1β as compared to control (Fig. 1B). Results

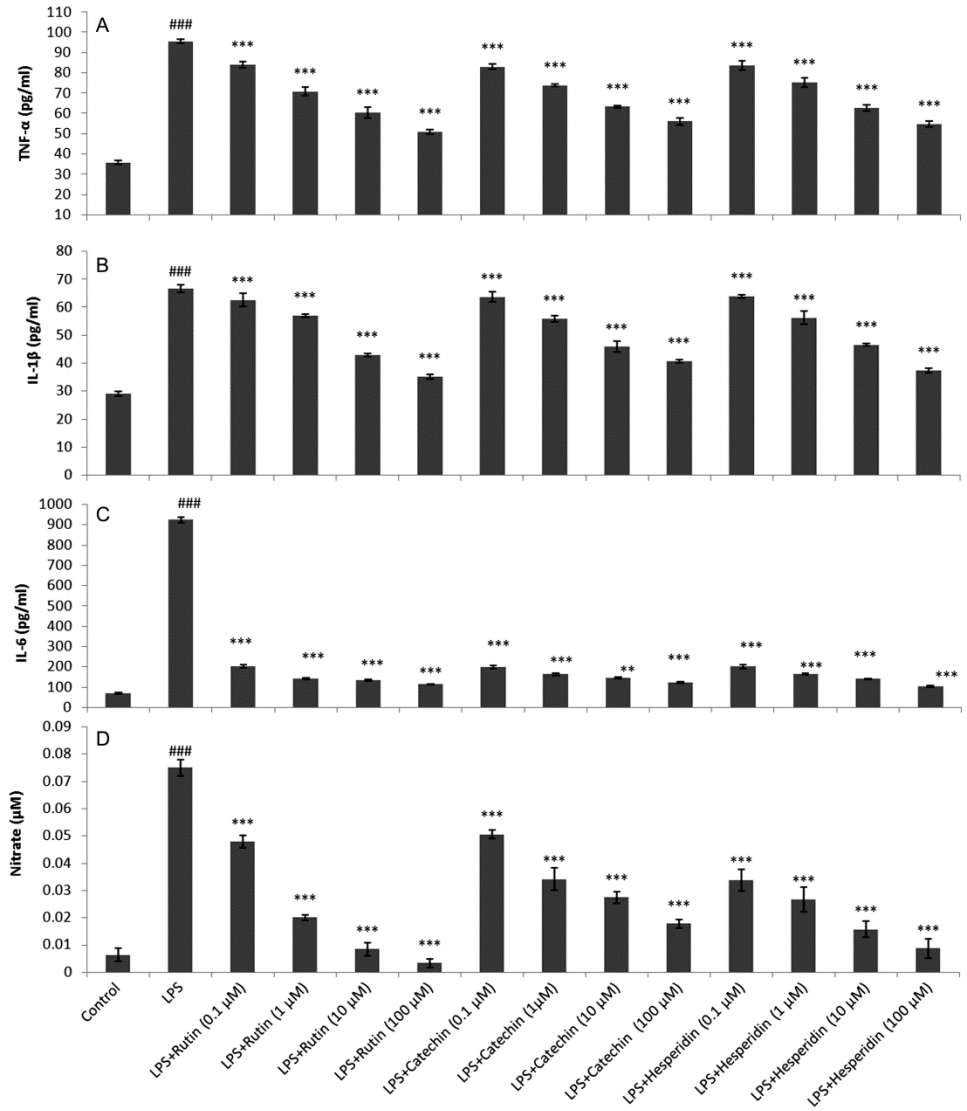


Fig. 1 — Effects of the rutin, catechin and hesperidin on the production of (A) TNF-α; (B) IL-1β; (C) IL-6; and (D) NO in rat macrophages. Rat peritoneal macrophages cells were cultured with indicated concentrations of catechin in combination with LPS (1 ng/mL) at 37°C in a 96-well plate. Media were collected after 6 h of treatment. Data are expressed as the mean ± S.D. of three individual experiments. Statistically significant change of cytokine release (*$P$ <0.05, **$P$ <0.01, ***$P$ <0.001), as compared with the group treated with LPS

indicated that all the tested flavonoids inhibited the release of IL-6 from macrophages in a concentration-dependent manner whereby maximum inhibition was observed at concentration 100 µM ($P < 0.001$) (Fig. 1C).

**Effect on NO production**

Because NO is also considered as a pro-inflammatory mediator in various acute and chronic inflammatory diseases, the present work was aimed to determine whether rutin, catechin, and hesperidin modulate the NO production from rat macrophages stimulated by LPS. The test flavonoids inhibited the NO production in a concentration-dependent manner at a dose range of 0.1-100 µM (Fig. 1D). The maximum inhibition was observed at 100 µM compared to control ($P < 0.001$).

## Discussion

The process of inflammation plays a key role in the development of various dreadful diseases *viz*. diabetes, cancer, and other immuno-disorders[24]. Many traditional medicines are documented to have an anti-inflammatory property and thus they may be beneficial to modulate the immune system[25]. Macrophages are among the important mediators of immunity and participate to play an important role in host's defense mechanism[26]. Macrophages are activated by stimuli that include phorbol esters, interferon γ, and lipopolysaccharides. Maintenance of homeostasis in the body is the prime function of macrophage. However, the phenomenon of immunostimulation due to macrophage over activity may add towards the progression of inflammatory and autoimmune diseases[27].

In the present work, immunomodulatory effect of rutin, catechin, and hesperidin was evaluated on macrophage activity. One of the important functions of macrophages is to secrete cytokines. Cytokines influence and initiate inflammation and causes the destruction of tissue leading towards 'inflammatory diseases'[28]. Interleukins and necrosis factors are associated with 'hyperalgesia'[29]. In the normal course TNF-α protects body against viral and bacterial infections. They do so by promoting the migration of neutrophils at the site of infection[30]. Due to the over activity of this cytokine, progression of inflammatory diseases is observed. Thus, the inhibition TNF-α seems to be beneficial in the management of inflammatory disorders. The treatment of macrophages with LPS resulted in the excessive production of TNF-α. In the present study, treatment with rutin, catechin, and hesperidin resulted in a significant fall in the level of TNF-α. The decrease in the concentration of IL-1β due to rutin has been identified previously in other cells/ tissues[31]. These results are in affirmation with the previous studies where levels of TNF-α were decreased due to hesperidin treatment in diabetic rats[32]. Interleukins are cytokines. IL-1β is secreted by monocytes, lymphocytes, neutrophils and macrophages, and is overexpressed during inflammation. They play an important role in intercellular communications with leukocytes. Activation of IL-1β is mediated by inflammasomes and caspase-I. Thus, the inhibition of secretion of this cytokine to a normal level could be a beneficial strategy for combating inflammatory disorders. The decrement in the secretion of IL-1β by hesperidin seems to be beneficial in the management of disorders like arthritis[33] and airway inflammation[34]. In the present study, treatment with hesperidin resulted in the decrement of IL-1β levels. The decrease in the concentration of IL-1β due to rutin has been identified previously in other cells/tissues[31]. The findings of the study are in unity with some previous research[35].

Along with IL-1β, IL-6 plays an important role in mediating immune responses, acute-phase responses, haematopoiesis, and inflammation. It is expressed by monocytes, macrophages, and fibroblasts. It is due to IL-6 receptor activation due to which inflammation and the autoimmune response is provoked and seems to be involved in the pathogenesis of rheumatoid arthritis[36]. The result of the present study reveals a decrease in the concentration of IL-6 which suggests the possible role of hesperidin in the management of inflammatory conditions and is in agreement with some other research[32]. The decrease in IL-6 levels due to treatment with rutin, catechin, and hesperidin could be one of the important reasons for its down regulation.

NO is a gaseous signaling molecule. It is also termed as endothelium-derived relaxing factor' and is synthesized by enzyme 'nitric oxide synthase'[37]. It is abundantly found in macrophages and monocytes at the site of infection or inflammatory disease[38]. The biosynthesis of nitric oxide prevents bacterial propagation and retards inflammation (due to suppression of proliferation of T cells)[39]. NO plays an integral role in the progression of inflammation. The oversecretion of NO is due to cytokine activated macrophages. It is one of the chief regulators of apoptosis and plays an important role in inflammation and associated inflammatory disorders.

The expression of TNF-α and IL-1 follows i-NOS production. Overproduction of nitric oxide and 'reactive nitrogen intermediates' is associated with the progression of many inflammatory diseases[39,40]. The discomfiture of this enzyme may play a vital role in anticipation of inflammation. The outcomes of the present study revealed the decrement in the production of NO synthesis which could be predisposed due to inhibition of the underlying enzyme (nitric oxide synthase). These results are in association with previous *in silico* studies[41–43].

## Conclusion

The outcomes of the present study provide sufficient evidence that rutin, catechin, and hesperidin possess *in vitro* immunomodulatory activity. These test flavonoids enhanced macrophage activity by increasing cell proliferation and inhibited LPS stimulated activities of rat macrophage by suppressing the production of TNF-α, IL-1β, IL-6, and NO levels. Because flavonoids showed a significant decrease in levels of TNF-α, IL-1β, IL-6 and NO (induced by LPS), it seems to note that food-stuff rich in rutin, catechin, and hesperidin could play a pivotal role in regulating immune functions. In conclusion, it is confirmed that rutin, catechin, and hesperidin possess immunomodulatory activity which may initiate the functioning of innate immunity by promoting macrophage proliferation and suppressing LPS-stimulated TNF-α, IL-1β, IL-6 and NO levels in rat macrophages. The mechanism by which these flavonoids affect immune-modulation needs to be explored more.

## Conflict of Interest

All authors declare no conflict of interest.

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