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Original Article

Protective Effect of Hesperidin on Humoral and Cell-mediated Immunity in Rat Model

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ABSTRACT

Context: Hesperidin, an important bioflavonoid, is abundantly found in various foodstuffs and can be regarded as an essential nutraceutical. Hesperidin has been acknowledged for its protective and beneficial effects on various aspects of biological system. **Objectives:** The present study was aimed to examine the effect of hesperidin on the regulation of the immune response in experimental animal models. **Materials and Methods:** The immunomodulatory effects of hesperidin were determined in rats using different experimental models including carbon clearance test, cyclophosphamide-induced neutropenia, neutrophil adhesion test, effect on serum immunoglobulin, neutrophil adhesion and indirect hemagglutination test. Hesperidin was administered orally at doses of 25, 50 and 100 mg/ kg p.o. Cyclophosphamide (30 mg/kg p.o.) was used as a standard immunosuppressant. **Results:** Hesperidin in all test doses demonstrated notable immunomodulatory responses in a sheep red blood cell challenge model. There was a refurbishment in the functioning of leucocytes in cyclophosphamide-treated rats and an increased clearance of carbon particles. The antibodytiter in the hemagglutination test showed increased levels of immunoglobulin. **Discussion and Conclusion:** The results of the present study indicate that hesperidin possesses sufficient potential for modulating immune activity by cellular and humoral mediated mechanisms.
Key words: Hesperidin, Immunity, Antibody, Myelosuppression, Immunoglobulin.

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INTRODUCTION

The immune system is a comprehensive network of a variety of 'immune cells' and specific 'mediators'. The coordination of these cells is responsible for producing a specific immune response.[1] The reaction of the immune system against any antigen is due to two interconnected networks that involve innate and adaptive immunity. The innate immune system identifies and selectively destroys invading microbes,[2,3] whereas, adaptive immunity aid in the development of memory cells that protect affecter cells against the repeated response to antigens.[4] The competence of immune system is solely based on the interaction of antigens with immune cells. There are multiple interrelated mechanisms to maintain homeostasis. The alteration in cellular homeostasis might be responsible for the progression of the inflammatory diseases. Medicines from plants have been used by humans since prehistoric times. In the present time, complementary medicines have achieved attention to treat various immunological disorders. The use of medicinal plants can serve as a continual source of pharmaceuticals. Polyphenols and flavonoids are classes of secondary metabolites produced by plants. They are abundantly found in leaves, stems, flowers, fruits and seeds.[5] They are named polyphenols due to the presence of multiple phenolic rings in their structure.[6] These compounds can be regarded as an important class of nutraceuticals that can prevent neurological and cardiovascular diseases.[7] Hesperidin, a flavanon, is a by-product of citrus cultivation. It is also termed as 'vitamin P.' It is known to prevent capillary permeability and fragility.[8] Deficiency of hesperidin is associated with abnormal leakiness of blood capillaries and pain along with weakness, aches and leg cramps. In a study, hesperidin supplementation aided in lowering edema/ swelling and prevents fluid accumulation.[9] Hesperidin has also been studied for its beneficial effects on certain organ system. Despite this, no systematic study about the protective effect of hesperidin on the cellular and humoral system has been performed. Therefore, present work aimed to determine immunomodulatory effects of hesperidin on the rat model.

MATERIALS AND METHODS

Animals

Wistar rats (180-200 g) of either sex were housed in polypropylene cages, maintained under standardized conditions (12 hr light/dark cycles, 28±2°C) 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 Institutional Animal Ethics Committee.

Chemicals

Hesperidin was purchased from Central Drug House, Mumbai, India. All the other chemicals used in the study were of analytical grade Hi-Media, Mumbai, India Central Drug House, Mumbai, India, Qualigens, Mumbai, India).

Carbon ink

Carbon ink suspension (Camel, India) was injected into the rats in a dose of 10 µl/g.

Antigen

Sheep red blood cells (SRBCs) were collected in to Alsevier's solution, washed with large volumes of sterile normal saline thrice and adjusted to a concentration of 5 x 10^9 cells/ml. The cells were used for immunization and challenge (for primary and secondary immune challenge).

Selection of dose

According to the studies performed by Hemanth Kumar *et al.*[10] the hesperidin in the dose of 25, 50 and 100 mg/kg of body weight was used in the study.

Animal group and dosing

Animals were divided into five groups with six animals in each group. The groups were treated as follows:

Group, I Distilled water 2 ml/kg
Group II Cyclophosphamide (30 mg/kg)
Group III Hesperidin (25 mg/kg)
Group IV Hesperidin (50 mg/kg)
Group V Hesperidin (100 mg/kg)

Immunomodulatory study protocols

Hypersensitivity reaction (delayed type)

On day 0, all groups were immunized by subcutaneous administration of 1 ml of SRBC cell suspension into the right hind footpad. On day 15, all groups were challenged by subcutaneously injecting 0.5 mL of SRBC cell suspension into the left hind footpad.[11] The thickness of the left hind footpad was measured after 24 and 48 hr of a challenge using Vernier calipers. The difference in the thickness of the right hind paw and the left hind paw was used as a measure of DTH reaction and was expressed as a mean percent increment in thickness/edema using the following equation:

DTH reaction=(left foot pad challenged with antigen-right footpad (control))/(left foot pad challenged with antigen) ×100

Carbon clearance test

The phagocytic activity of the reticulo-endothelial system (RES) was assessed by the carbon clearance method. Briefly, 1 ml of Indian ink was administered intravenously to all five groups of adult Wister rats on the 8th day of daily administration of hesperidin (25, 50 and 100 mg/ kg). Blood samples were collected at 0 and 15 min intervals and transferred directly into the centrifuge tube, allowed to coagulate at room temperature and centrifuged at 2,000 rpm for 10 min. Then, 50 µL of clear supernatant (serum) was collected and transferred to the different volumetric flask. The volume was made up to 25 mL by distilled water; absorbance was measured at 650 nm.[12,13]

The phagocytic index K was calculated using the following equation:

$K = (\text{Loge } OD_1 - \text{Loge } OD_2)/15$

where OD_1 and OD_2 are the optical densities at 0 and 15 min respectively.

Cyclophosphamide-induced neutropenia

The method described by Manjrekar *et al.*[14] was adopted. The animals of groups II-V were injected with cyclophosphamide (100 mg/kg, i.p.) on the 11th, 12th and 13th day, one hr after the administration of the respective drug treatments. Blood samples were collected from retro-orbital plexus on the 14th day of the experiment. Determination of total white blood cells was carried out.

Neutrophil adhesion test

The rats were treated orally with standard and test compounds as mentioned above for 14 days. On day 14, blood samples were collected from the retro-orbital plexus into heparinized vials and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC). After the initial counts, blood samples were incubated with 80 mg/ml of nylon fibers for 10 min at 37°C. The incubated blood samples were again analyzed for DLC. The percentage of neutrophils in the treated and untreated blood was determined and the difference was taken as an index of neutrophil adhesion.[15,16]

The product of total leukocyte count and % neutrophil (known as the neutrophil index) was determined for each of the respective groups.[17] The % neutrophil adhesion for each of the test groups was determined as follows:

% Neutrophil adhesion=(difference in neutrophil count in untreated and fiber treated blood)/(neutrophil count of untreated blood) ×100

Indirect hemagglutination test

Rats were pretreated with the test drugs for 14 days and each rat was immunized with 0.5×109 sheep red blood cells (SRBCs) intraperitoneally, including the control rats. The day of immunization was referred to as day 0. The drug treatment was continued for 14 more days and blood samples were collected from each rat at the end of the drug treatment. The titer value was determined by titrating serum dilutions with SRBC (0.025×109 cells) in micro titer plates. The plates were incubated at room temperature for 2 hr and examined visually for agglutination. The minimum volume of serum showing hemagglutination was expressed as hemagglutination (HA) titer.[18]

Serum immunoglobulin

The five groups of rats were treated with standard/ test drug orally for 21 days. Six hours after the last dose, blood samples were collected and the serum was separated by centrifugation, the collected serum was used for estimation of immunoglobulin levels. Briefly, for each serum sample, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulfate solution was prepared. To each, 0.1 ml of serum was added and inverted to enable complete mixing of the reagents. The tubes were allowed to stand for 1 h at room temperature. The first tube served as blank. The turbidity developed was measured spectrophotometrically at 580 nm. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulfate ($BaSO_4$) solution. The standard $BaSO_4$ solution was prepared by adding 3 ml of barium chloride solution (1.15% w/v) to 97 ml of 0.2 N sulphuric acid. The turbidity obtained with this solution was expressed as 20 zinc sulfate turbidity (ZST) units.[19]

Statistical analysis

All the values were expressed as mean ± SEM. Statistics was applied using Graph Pad Prism version 5.0 for Windows, Graph Pad software, San Diego, California, USA. One-way ANOVA followed by Dunnet's comparison test was used to determine the statistical significance between various groups. Differences were considered to be statistically significant when $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

Hypersensitivity reaction

The delayed type response against SRBC in rats (that demonstrates 'cell-mediated immunity') displayed a 'dose-dependent' augmentation owing to treatment with hesperidin. Hesperidin in the dose of 25, 50 and 100 mg/ kg/day demonstrated foot pad thicknesses of 4.91±0.07 mm, 5.01±0.10 mm and 5.36±0.08 mm respectively after 24 hr. The corresponding value of 4.68 ± 0.16 mm was obtained for group II. The differences in DTH response were statistically significant ($^{***}p<0.001$,$^{\#\#\#}p<0.001$) for a dose of 25, 50 and 100 mg/kg (Figure 1).

Carbon clearance test

Hesperidin administration (especially in the dose of 100 mg/ kg), caused increased clearance of carbon particles from the blood, which was evident by a significant increase in the phagocytic index ($^{***}p < 0.001$; $^{\#\#\#}p < 0.001$). Administration of cyclophosphamide caused a decrease in the phagocytic index (Figure 2).

Cyclophosphamide induced neutropenia

Hesperidin administration in a dose of 25, 50 and 100 mg/kg/day caused a decrease in neutropenia which is reflected by a significant increase in total leukocyte count (***p<0.001, ###p<0.001). Administration of cyclophosphamide resulted in a decrease in the leukocyte count (Figure 3).

Neutrophil adhesion

Incubation of neutrophils with nylon fibers resulted in a decrease in the neutrophil counts which was related to adhesion of neutrophils to the fibers. Administration of hesperidin caused a significant increase in the neutrophil adhesion (***p<0.001; ###p<0.001) compared to the control. The lower dose of hesperidin was more effective than the high dose (Table 1).

Hemagglutination reaction

Hesperidin administration demonstrated a significant increase in the antibody titer at low dose levels. The dose of 25 mg/kg/day showed a response of 106.66 ± 13.49 (**p<0.01) and the dose of 50 mg/kg/day showed a response of 138.66 ± 10.66 (***p<0.001) in comparison to group I (192.00 ±28.62). There was a significant change in humoral antibody level titer levels in animals treated with cyclophosphamide (***p<0.001; 53.33 ± 16.52), (Figure 4).

Serum immunoglobulin

As evident from Figure 5, a significant increase in the serum immunoglobulin levels in the hesperidin treated group (group III 10.12 ±0.38, ***p < 0.001; group IV 11.19 ±0.39, ***p < 0.001, ###p < 0.001; group V 13.01±0.28, ***p < 0.001, ###p < 0.001) was seen, whereas group II animals revealed lower serum immunoglobulin levels (8.61±1.22) compared to vehicle control group I animals (14.59±0.48).

DISCUSSION

The immune system functions to safeguard the body against invading pathogens, foreign bodies and tumor and thus plays a noteworthy role in maintaining 'homeostasis in the body. The process of modulation of immune system, either by stimulating or suppressing its functions aids to sustain disease-free state in living beings. There are many situations where immuno-suppression is required to overcome undesired immuno-potentiation. Similarly, immunostimulation is needed to overcome drug-induced/auto immune-malfunctioning. Immunomodulation due to natural products seems to be a promising strategy. This can afford a support for activation of host defense mechanism in case of impaired immune response. In the present times, phytochemical have been explored for potential immunomodulatory effects.[20–26]safeguard and proper functioning of immune system. Ruin, an important bioflavonoid, is abundantly found in various foodstuffs. Rutin has been acknowledged for its protective and beneficial effects on various aspects of the biologi-

Table 1: Effect of Hesperidin administration on leucocyte mobilization/ migration in rats.

Treatment	TLC		% Neutrophils		Neutrophil Index		% Neutrophil adhesion
	UTB	TB	UTB	TB	UTB	TB	
Group I	6.20±0.12	5.64±0.10	45.50±1.05	36.66±1.38	282.46±12.68	206.83±14.65	9.13±0.14
Group II	4.76±0.28	4.40±0.25	40.66±0.49	34.82±0.65	193.81±13.89	153.54±16.81	7.51±0.24***
Group III	6.14±0.17	5.03±0.42	46.33±0.56	36.66±0.42	284.51±13.26	184.71±1.82	17.95±0.20***
Group IV	6.33±0.18	5.46±1.19	49.16±0.65	38.16±1.20	311.35±11.60	208.64±14.28	13.68±0.22***
Group V	6.41±0.08	5.65±1.15	50.83±0.87	39.66±1.50	326.16±7.46	224.41±22.50	11.81±0.36^{ns}

Results are given as mean ± SEM of six animals in each group. Group I compared with rest of the treated groups. Significance at *p < 0.05; **p< 0.01; ***p < 0.001.

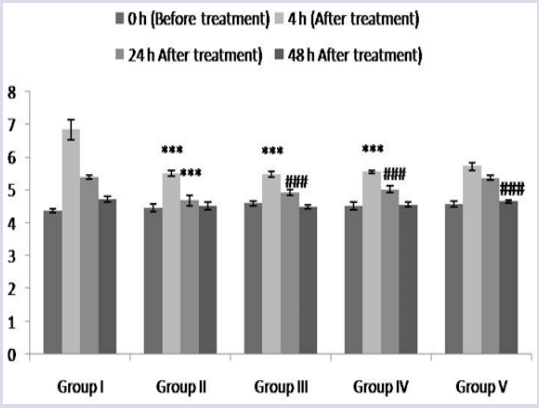

Figure 1: Effect of Hesperidin administration on delayed type hypersensitivity response in rats. Results are given as mean ± SEM of six animals in each group. Significance at *p < 0.05; **p< 0.01; ***p < 0.001 when compared to Group I; #p < 0.05; ##p< 0.01; ###p < 0.001 when compared to Group II

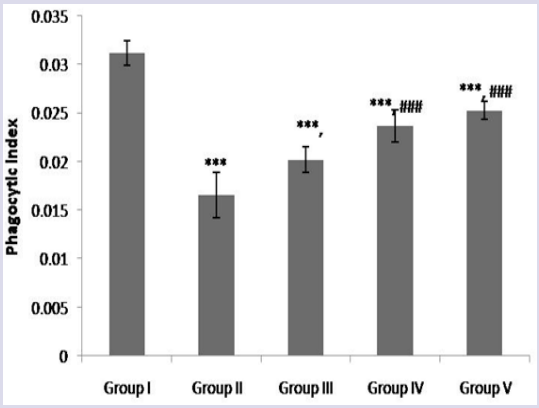

Figure 2: Effect of Hesperidin administration on phagocytic index in rats. Results are given as mean ± SEM of six animals in each group. Significance at *p < 0.05; **p< 0.01; ***p < 0.001 when compared to Group I; #p < 0.05; ##p< 0.01; ###p < 0.001 when compared to Group II

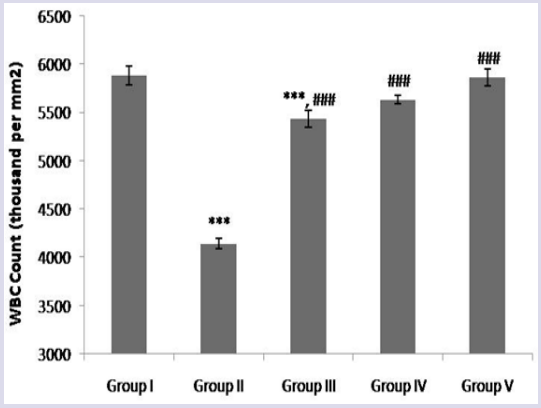

Figure 3: Effect of hesperidin administration on WBC counts in rats. Results are given as mean ± SEM of six animals in each group. Significance at *p < 0.05; **p< 0.01; ***p < 0.001 when compared to Group I; #p < 0.05; ##p< 0.01; ###p < 0.001 when compared to Group II.

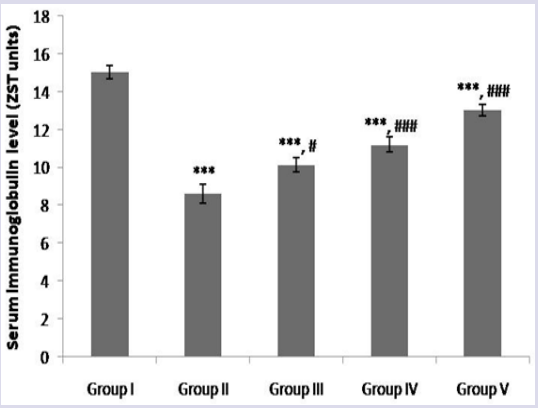

Figure 5: Effect of Hesperidin administration on serum immunoglobulin levels in rats. Results are given as mean ± SEM of six animals in each group. Significance at *p < 0.05; **p< 0.01; ***p < 0.001 when compared to Group I; #p < 0.05; ##p< 0.01; ###p < 0.001 when compared to Group II.

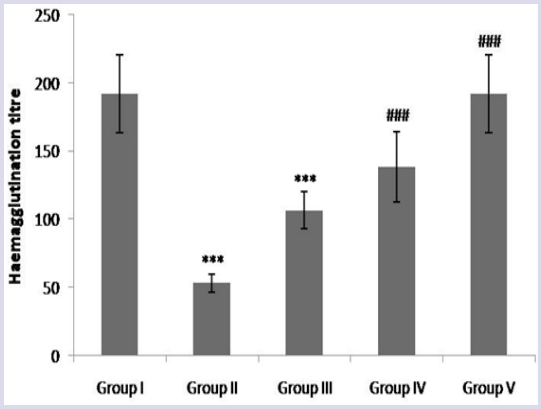

Figure 4: Effect of hesperidin administration on HA antibody titer in rats. Results are given as mean ± SEM of six animals in each group. Significance at *p < 0.05; **p< 0.01; ***p < 0.001 when compared to Group I; #p < 0.05; ##p< 0.01; ###p < 0.001 when compared to Group II.

cal system. The present study was aimed to examine the effect of rutin on the regulation of the immune response in experimental animal models. Effect of rutin of cellular immunity was determined by delayed-type hypersensitivity (DTH The present study demonstrates the protective effect of hesperidin on cellular and humoral immunity in rats.

In the present study, the doses of hesperidin 25, 50 and 100 mg/ kg)[10] was used. Delayed-type hypersensitivity reaction is a cell-mediated immune event which requires about two days to develop. This reaction can be experimentally induced by various allergens viz. 2,4-dinitrofluorobenzene, oxazolone and sheep red blood cells (SRBCs).[27] In this reaction, T-cells are employed by tissue where they localize followed by activation of antigen-presenting cells. This event promotes the production of cytokines (especially interleukin 3) that produces local inflammation.[28] DTH induced by SRBCs is a 'tuberculin-type' reaction. The T-cells are evoked and the immunological response is observed after 18-24 hr of SRBC challenge. The reaction progresses with time and declines after 48-96 hr.[29] This process is feature of the chronic inflammation process. The situation like rheumatoid arthritis indicate DTH in the cell. In the present study, administration of hesperidin in rats caused a significant increase in the delayed type of hypersensitivity (Group III, IV and V; ***p < 0.001; ###p < 0.001). Such an effect could be due to the stimulatory effect of hesperidin on 'chemo taxis-dependent leucocytes migration.'

The carbon clearance test is one of the important tests that evaluate the capability of test compounds to preserve the vitality of reticuloendothelial system. The reticulo-endothelial system is a diffused type system that is progressed due to phagocytosis cells. These cells play a leading role in the removal of foreign particles and dead cells from circulation. Once the colloidal particles are injected into blood, the process of phagocytosis is initiated for the removal from the blood stream.[30] In the present study, prior treatment with hesperidin to animals caused an increment in phagocytic activity which could be directly allied with augmented production of phagocytic cells.[27] The phagocytic index of Group IV and V animals was significantly more compared to the cyclophosphamide-treated group (Group II).

Cyclophosphamide is one of the important classes of chemotherapeutic drugs that is used in the treatment of carcinoma. However, it suffers from major drawbacks, which includes the suppression of leukocyte growth and maturation (myelosuppression). This condition is observed by a reduction in cellularity of bone marrow and a decrease in the content of stem cells as well as progenitor cells. 'The growth of granulocyte-macrophage progenitors is also suppressed.[31,32] Some CIT regimens are not well tolerated by many patients >or=70 years of age.\\n\\nMETHODS: Sixty-four previously untreated patients with CLL and serum creatinine <1.5 times the upper limit of normal who met National Cancer Institute (NCI Aldophosphamide, a metabolite of cyclophosphamide is also cytotoxic and increases oxidative stress within the cell. Thus, the phenomenon of immunosuppression occurs. In the present study, cyclophosphamide treatment in animals (Group II) resulted in persistent neutropenia. However, administration of hesperidin to animals (Group III-V) resulted in a significant increment (***p < 0.001; ###p < 0.001) in the levels of white blood cells. Such an effects could be due to the cytoprotective[33,34] and antioxidant[35] effect of hesperidin over the biological system.

Neutrophils are an integral part of 'cell-mediated immunity.' They aid with thev removal of harmful pathogens and foreign bodies.[36] In this study, hesperidin administration caused a significant increase in percent neutrophil adhesion. Such an effect suggests the involvement of antioxidant effects. Adhesion of neutrophils to nylon fibers represents the

movement of neutrophils towards the site of 'inflammation'.[16] One of the possible reasons for such an effect could be an increase in the activity of 'β2 integrins' that are present on the neutrophil surface, which adheres to nylon fibers.

In immunomodulatory studies, the indirect haemagglutination test is helpful to determine the effect of test compounds on B cell activation and expression. The SRBC surface comprises a unique antigen known as 'foreman antigen' (Forssman heterophilic glycolipid antigen). It is a glycosphingolipid with 'antaigenic specificity' and responsible for humoral activation in rats.[37] Phytoconstituents are reported to enhance production of antibody against this antigen.[20,23,38,39] Antibody titer values of animals treated with hesperidin (Group III, IV and V;$^{***}p < 0.001$; $^{###}p < 0.001$) were higher compared to Group I and Group II animals. Thus, the increased response to SRBCs due to hesperidin demonstrates increased sensitivity of B cells to the production of antibody. In addition to HA titre studies, production of antibodies due to SRBC challenge was also determined by zinc sulfate turbidity method. Incorporation of zinc sulfate to serum rich in immunoglobulins causes their precipitation making the solution cloudy. The absence of cloudiness in serum treated with zinc sulfate solution embodies a lack of immunoglobulins.[40] A notable increase in the levels of serum immunoglobulin production by hesperidin was seen in the present study. Thus, the study reflected the stimulation in production of Ig G.

CONCLUSION

The results of the present study established that hesperidin in the test doses increased cellular and humoral immunity in rat model. Hesperidin decreased cyclophosphamide induced myelosuppression. Due to hesperidin supplementation, there was an increased reduction in the levels of foreign particles, an increase in antibody titre and the levels of immunoglobulins. Results of the present study establish hesperidin to be an immunomodulatory agent. Additional studies on hesperidin are necessary to establish its therapeutic effects on immune depressing state and autoimmune diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CD: Cluster for differentiation; **SRBCs:** Sheep red blood cells; **RES:** Reticuloendothelial system; **TLC:** Total leukocyte count; **DLC:** Differential leukocyte count; **ZST:** Zinc sulfate turbidity.

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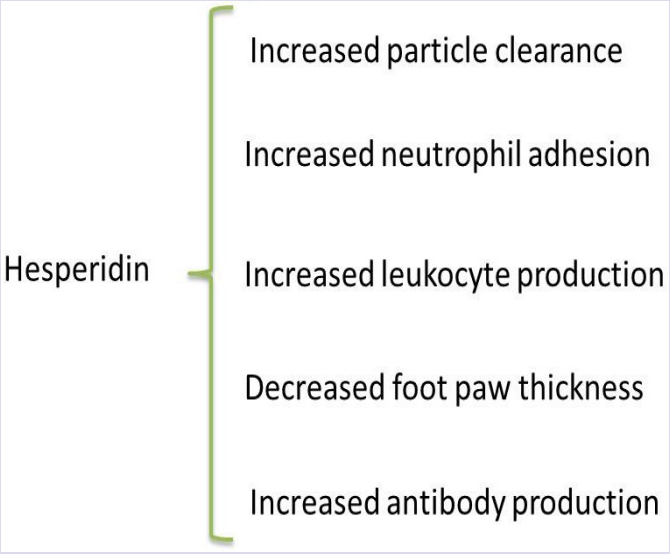
PICTORIAL ABSTRACT



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