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Immunomodulating Effects of *Allium sativum* in Renal Tissue of LPS Challenged Mice

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Abstract

Allium sativum contain various medicinal properties like immunomodulatory, antitumorigenetic, antiatherosclerotic and antimicrobial. In the present study, we analyzed the impact of hydroethanol extract of *Allium sativum* on cytokine production in LPS challenged mice. LPS significantly induced the activation of NF-kB and also increased the levels of NO, TNF-α, IL-6, IFN-Y, PGE2 with the suppression of IL-10 production. In the results we found that *Allium sativum* significantly attenuated the LPS-induced phosphorylation of NF-κB and normalized the levels of NO, TNF-α, IL-6, IFN-Y, PGE-2 and IL-10. In summary, it was deduced that garlic may promote an anti-inflammatory effect by inhibition of NF-kB activity in the tissue.

Keywords: *Allium sativum,* Anti-inflammatory, Cytokines, Lipopolysaccharide (LPS), NF-kB

1. Introduction

Innate immune cells like macrophages identify the existence of invading bacteria and begin host response by discharging cytokines and chemokines. LPS, apro-inflammatory endotoxin, is found in outer layer of all gram-negative bacteria. When bacteria multiply in the body, LPS is released and recognized by various circulating cell types, triggering the activation of NF-κB-dependent pro-inflammatory cytokines such as Tumornecrosis Factor-α (TNF-α), Interleukins (IL), prostaglandins and nitric oxide. However, excess cytokine production can have deleterious reactions. Septic-shock triggered by LPS causes' the formation of reactive oxygen species and multi-organ damage[1–3]. In ayurvedic remedy, garlic is used to reduce many health problems such as respiratory, circulatory and excretory disorders. The first chemically isolated compound of the garlic is Allicin, which contains antiviral, antibacterial and antifungal activities. Previous reports showed that pharmacological potency of garlic which contains antioxidant, hypolipidemic, hepatoprotective, anti-tissue, atrophy, antidiabetic antigenotoxic and immunomodulatory activities, the active constituents of garlic antioxidants includes organosulfur compounds, flavonoids such as allicin, trace elements and other bio-active constituents. Garlic can directly modulate cytokine production in lymphocytes and largely recognized as having anti-inflammatory properties[4,5]. Swiss albino mice is a mammalian model and genetically more similar to humans than to other experimental model which has short gestation period, high fertility rate, convenient body size and easily procurable were selected for the proposed study.

So, therefore keeping the above medicinal properties of garlic the current study is designed to examine its possible protective role on inflammatory parameters and also to observe the various pathophysiological events associated with toxicity in renal tissue of swiss albino mice exposed to LPS.

2. Material and Methods

2.1 Chemicals

All chemicals were of analytical grade and procured from reputed laboratories and companies (Invitrogen thermo-fisher scientific ELISA kits).

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2.2 Experimental Plant

The bulb of the experimental plant i.e. *Allium sativum* was collected from the local region of Banasthali Vidyapith, Rajasthan. Bulb of *Allium sativum* was shade dried and powder was used for extraction. Soxhlet was used for hydroethanol extraction of plant material. Extracted mixture was evaporated at 40°C, using rotatory evaporator and stored in air tight container.

2.3 Animals

The experimental animal model i.e. male swiss albino mice (*Musmusculus*) weighing between 15-25 g were procured from Lala Lajpat Rai University of Veterinary and Animal Science, Hissar, India, (Ref. No. BU/BT/402/14-15). Adult male swiss albino mice were cared in a well-ventilated animal room with 12 h light-dark cycle. They had continuous access to water and dry pellet diet and had access to drinking water ad libitum. Metal contents in parts per million (ppm) dry weights in commercial pelleted diet are as follows: Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0 and Fe 75.0. Essential cleanliness and sterile conditions were maintained to ensure hygienic and pathogen-free environment.

2.4 Experimental Groups of the Animals for the Post Treatment

In the current investigation, 30 swisses albino mice weighing approximately 25–30 g were divided into five groups having six mice in each were used to study renal oxidative stress and biochemical parameters. Group 1 served as control having six healthy subjects and was given normal saline. Group 2 served as LPS treated having 24 male mice which were given 2 mg/kg of body weight, i.p. dose for 14 days. After, 14 days the plant extract low dose, high dose and standard dose were given to the animals from 15th day and continued to 21st day. Thus, the total duration of the treatment given was of 21 days. The groups are tabulated below:

2.5 Experimental Groups of Animals for the Simultaneous Treatment

Animal were divided into 8 groups; each group was having six animals. Total mice required for simultaneous treatment were 48. The whole duration of treatment was of 21 days. The Group 1 served as control and received saline. Group 2 mice were exposed to LPS (2 mg/kg b.w.). Groups from 3rd to 5th were given single dose of *Allium sativum* low dose, *Allium sativum* high dose and Standard dose. Group 6 and 7 mice were treated with LPS + *Allium sativum* low dose (100 mg/kg b.w.) and LPS+*Allium sativum* high dose (300 mg/kg b.w.) respectively. Group 8 mice were received LPS+standard dose (Dexamethasone). The groups are tabulated below:

2.6 Homogenate Preparation

After giving both post and simultaneous treatments to animals, they were sacrificed by cervical dislocation. Kidneys tissues were removed and rinsed for cleaning in cold saline. After cleaning they were blotted dry then weight was taken. Kidneys tissue was cut into small pieces and kept in ice chilled 0.1 M (pH 7.4) sodium phosphate buffer and homogenate was prepared in mortar pestle. After this homogenate was centrifuged at 10,000 rpm at 4°C for 15-20 min. After centrifugation supernatant was used as homogenate.

2.7 Inflammatory Markers

Nf-kB transcription activity, PGE2 level, Interleukin-6 level,Interleukin-10 level, Iterferon- gamma, Tumor necrosis factor alpha were determined using commercially available enzyme immunoassay kit (Invitrogen thermo-fisher scientific ELISA kits) and Nitric oxide level were determined by the method of Green *et al.*,[6].

Groups	N = 6 in Each Group	Treatments
Group 1	Control	Normal Saline
Group 2	LPS Treated	2 mg/kg b.w
Group 3	LPS + *Allium sativum* low dose	LPS (2 mg/kgb.w) + Hydroethanol extract of *Allium sativum* low dose; 100 mg/kg b.w.
Group 4	LPS+ *Allium sativum* high dose	LPS (2 mg/kgb.w) + Hydroethanol extract of *Allium sativum* high dose; 300 mg/kg b.w.
Group 5	LPS+ Standard (Dexamethasone)	2 mg/kg b.w. and 10 mg/kg b.w. respectively

2.8 Histopathological Analysis

In kidney tissue of experimental animals (Post Treatment) cytotoxicity and histopathology analysis has been done by the method of Manus and Mowry[7],

2.9 Statistical Analysis

All results were performed as ± standard deviation. The results were subjected to one way Analysis of Variance (ANOVA), and analysis with $p<0.05$ were considered as statistically significant and $p<0.001$ as very satisfactory.

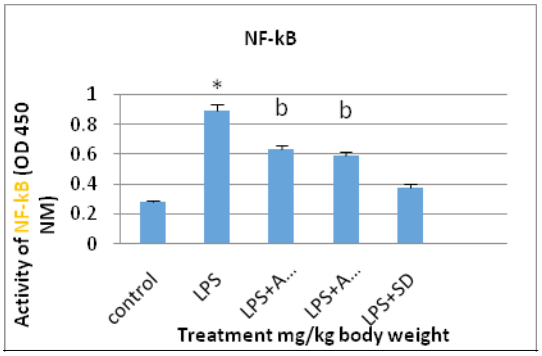
3. Results

3.1 *Allium sativum* Attenuates LPS-induced NF-kB Activation

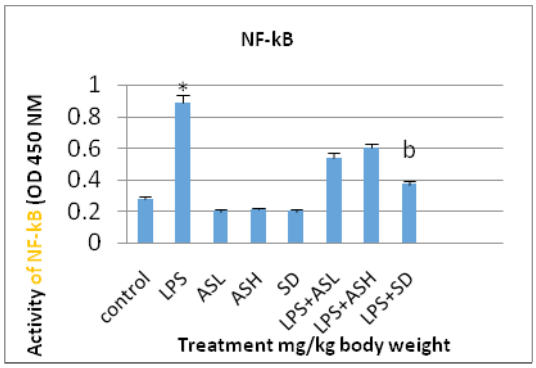
To determine the anti-inflammatory effects of *Allium sativum*, the activation of key molecule NF-κB, in forming cytokine expression, was analyzed in the current study. In the results it was found that LPS caused increase the activation of NF-κB. Quantitative measurements showed that *Allium sativum* significantly attenuated the LPS-induced NF-κB phosphorylation.

3.2 Effect of *Allium sativum* on Renal TNF-α, IL-6, IL-10, IFN-Y and PGE2 levels in LPS-treated Mice

Effect of *Allium sativum* on Renal TNF-α, IL-6, IL-10, IFN-Y, PGE2 were determined by ELISA assay as shown in Figure 2. LPS induced increase in the levels of TNF-α, IL-6, IFN- and PGE2 which were suppressed by *Allium sativum*. LPS toxicity suppressed the level of IL-10 as compared to control mice. Suppression of IL-10 promotes the enhancement of TNF-α formation endogenously. *Allium sativum* significantly prevented the decline in endogenous levels of IL-10 in renal tissue.



(A)

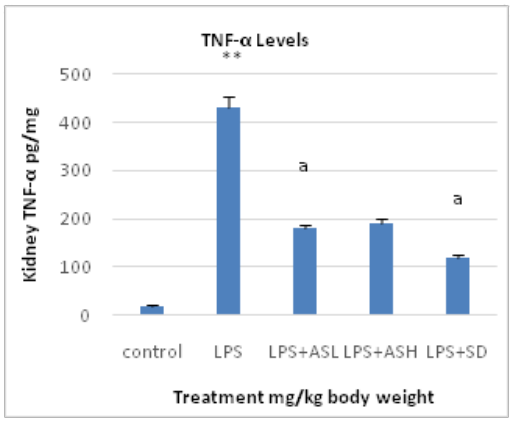


(B)

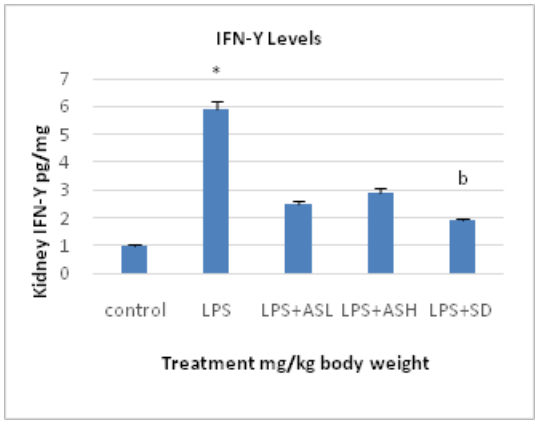
Figure 1. Effect of *Allium sativum* on LPS-induced changes in the levels of NF-kB activation. **(A)** NF-kB (OD in 450) in post treatment and **(B)** NF-kB (OD in 450) in simultaneous treatment in kidney tissues of male swiss albino mice were injected with 2 mg/kg LPS. SD - Standard dose; ASL – *Allium sativum* low dose; ASH - *Allium sativum* high dose. Values are expressed as mean±SD (n = 6). *$p<0.001$, **$p<0.05$ v/s control group (C); a$p<0.001$, b$p<0.05$ vs. compound treated group (LPS).

Group Number	N = 6 in Each Group	Treatments
Group 1	Control	Normal saline
Group 2	Compound treated (LPS)	2 mg/kg b.w.
Group 3	*Allium sativum* low dose	100 mg/kg b.w.
Group 4	*Allium sativum* high dose	300 mg/kg b.w.
Group 5	Standard dose	10 mg/kg body weight
Group 6	LPS + *Allium sativum* low dose	LPS (2 mg/kg b.w) + Hydroethanol extract of *Allium sativum* low dose; 100 mg/kg b.w.
Group 7	LPS + *Allium sativum* high dose	LPS (2 mg/kg b.w) +Hydroethanol extract of *Allium sativum* high dose; 300 mg/kg b.w.
Group 8	LPS + standard dose (Dexamethasone)	LPS (2 mg/kg b.w) +2 mg/kg b.w. and 10 mg/kg b.w.

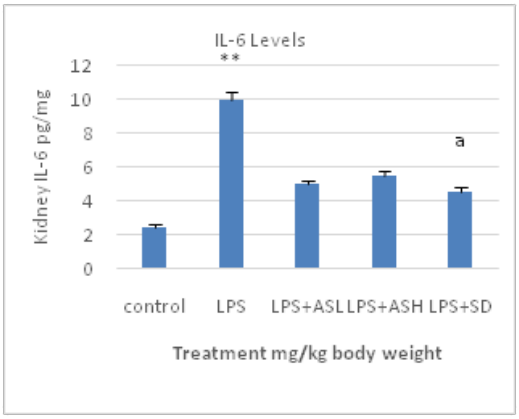
3.3 Effect of *Allium sativum* (Post Treatment) on Renal TNF-α, IL-6, IL-10, IFN-Y and PGE2 levels in LPS-treated Mice



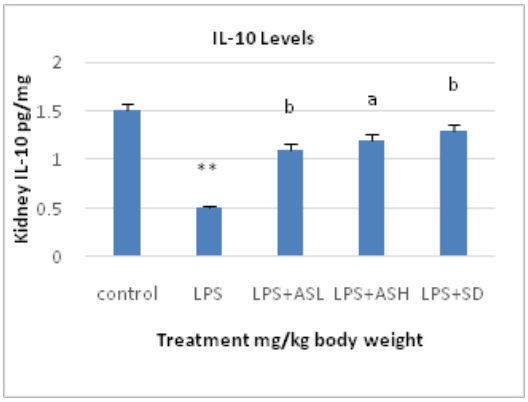
(A)



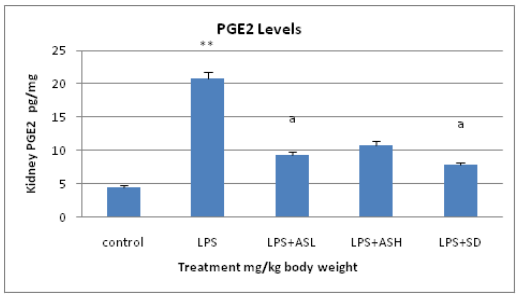
(B)



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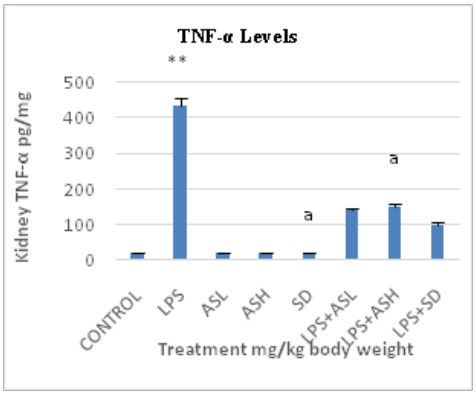
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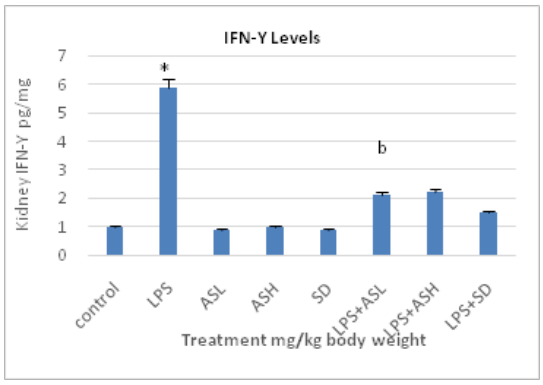
(E)

Figure 2. Effect of *Allium sativum* (post treatment) on LPS-induced changes **(A)** TNF-α, **(B)** IFN-Y, **(C)** IL-6, **(D)** IL-10 and **(E)** PGE-2 in kidney tissues of male swiss albino mice. SD- Standard dose; ASL – *Allium sativum* low dose; ASH - *Allium sativum* high dose. Values are expressed as mean±SD (n = 6). *p<0.001, **p<0.05 vs. control group (C); ap<0.001, bp<0.05 vs. compound treated group (LPS).

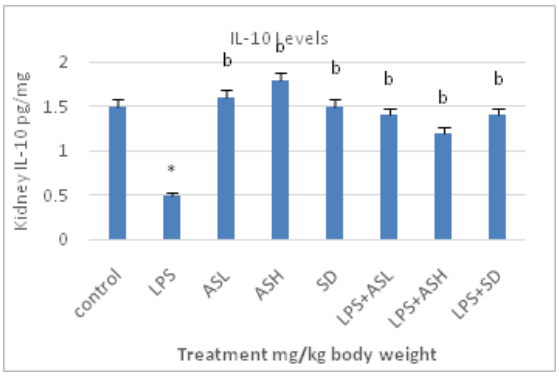
3.4 Effect of *Allium sativum* (Simultaneous Treatment) on Renal TNF-α, IL-6, IL-10, IFN-Y and PGE2 levels in LPS-treated Mice



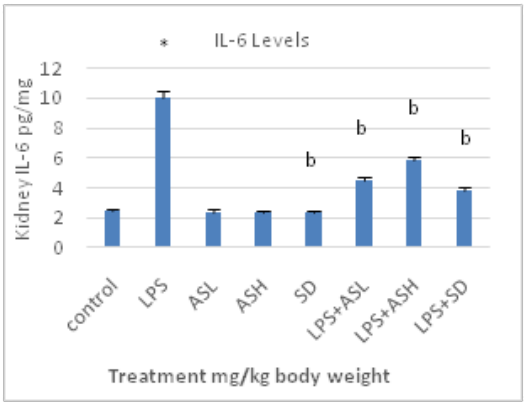
(A)



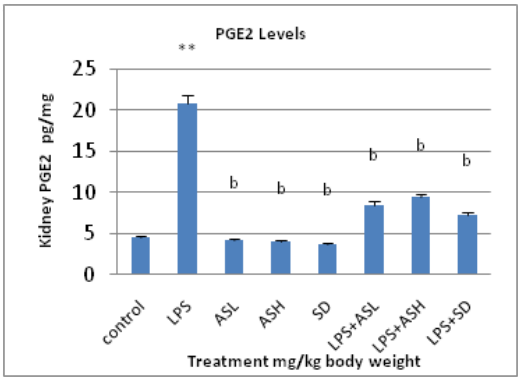
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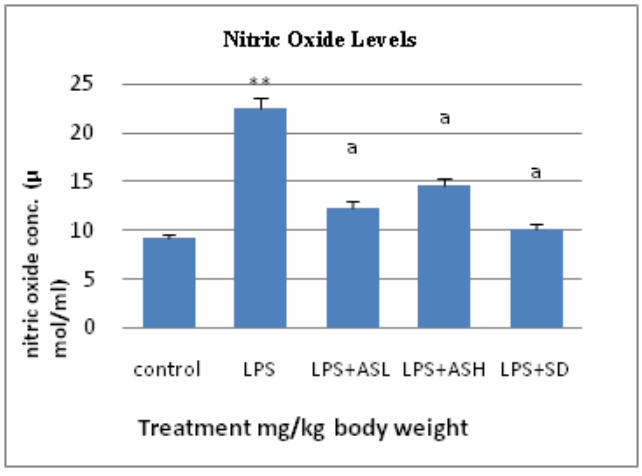


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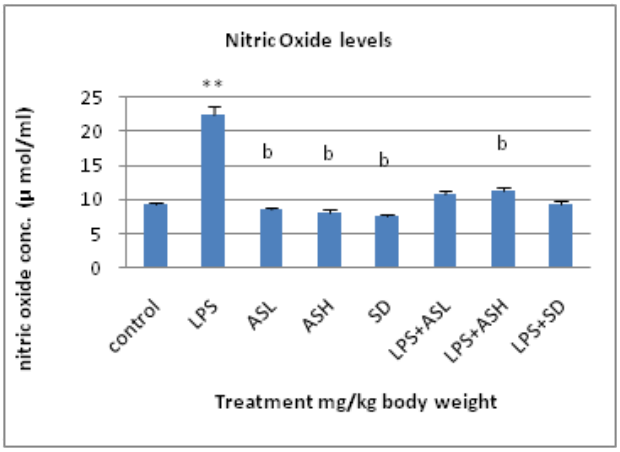


(E)

Figure 3. Effect of *Allium sativum* (simultaneous treatment) on LPS-induced changes in **(A)** TNF-α, **(B)** IFN-Y, **(C)** IL-6, **(D)** IL-10 and **(E)** PGE-2 in kidney tissues of male swiss albino mice. SD - Standard dose; ASL – *Allium sativum* low dose; ASH - *Allium sativum* high dose. Values are expressed as mean±SD (n = 6). *p<0.001, **p<0.05 vs. control group (C); ap<0.001, bp<0.05 vs. compound treated group (LPS).



(A)



(B)

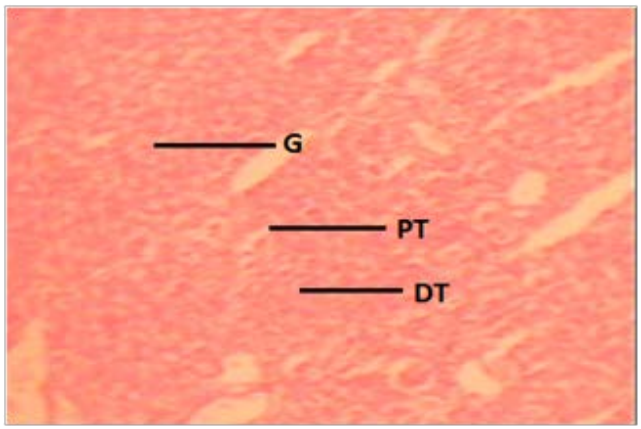
Figure 4. Effect of *Allium sativum* on LPS-induced changes in levels of nitric oxide. **(A)** Nitric oxide level in post treatment and **(B)** Nitric oxide level in simultaneous treatment in kidney tissues of male swiss albino mice were injected with 2 mg/kg LPS. SD - Standard dose; ASL – *Allium sativum* low dose; ASH - *Allium sativum* high dose. Values are expressed as mean±SD (n = 6). *p<0.001, **p<0.05 vs. control group (C); ap<0.001, bp<0.05 vs. compound treated group (LPS).

3.5 Effect of *Allium sativum* on Renal Nitric Oxide Level in LPS Treated Mice

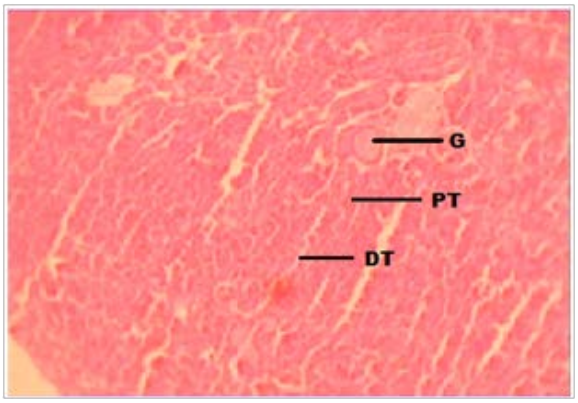
The Griess reaction was used to assay NO metabolites. Results showed the significant effect of LPS on NO metabolites concentrations in the renal tissues. NO metabolites concentrations in the renal tissues of LPS challenged mice were higher than control group. *Allium sativum* decreased the concentrations of these metabolites in compared with LPS group.

3.6 Histological Analysis

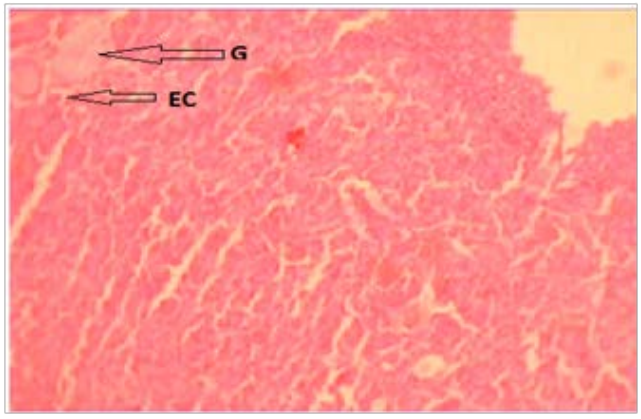
The histological changes in tissue sections of kidneys are shown in Figures A to E. The tissue sections of kidney of control animals showed normal renal architecture whereas in the LPS-induced mice, showed degeneration of renal tubular epithelial cells, blood vessel congestion and vast tissue damage by glomerular and tubular congestion with abnormality in Bowman's capsule[8]. However, *A. sativum* has shown significant effect at both dose levels when compared with LPS-induced group, with low damaged area and less extent of congestion. The low dose (100 mg/kg b.w.) of *Allium sativum* was found to be less effective in recovering the normal architecture of renal tissues and high dose (300 mg/kg b.w.) of *Allium sativum* was found to be relatively effective as it recovered the normal glomerular and tubular organization from the degeneration of renal tubular epithelial cells and blood vessel congestion.



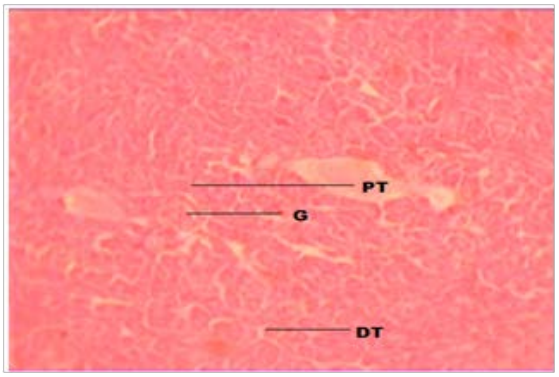
(A). CONTROL-Normal saline treated. Showing normal glomerulus (G), Proximal (PT) and distal (DT) tubules.



(C) **LPS + *A. sativum* (100mg/kg body weight)**
The low dose of *Allium sativum* was found to be less effective in recovering the normal architecture of renal tissues.

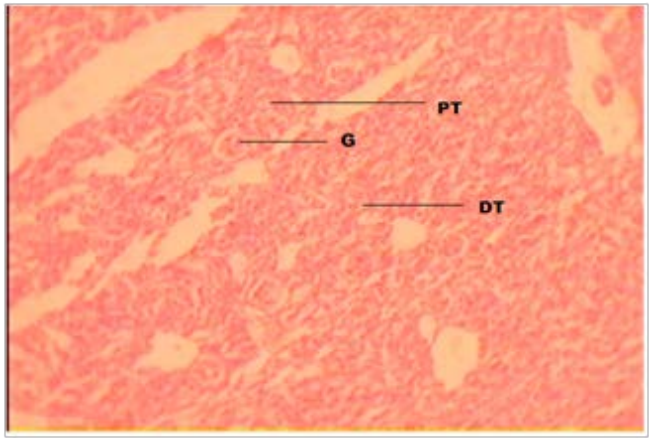


(B). LPS treated 2mg/kg body weight. LPS-treated mice kidney tissues, histological analysis has shown degeneration of tubular epithelial cells (ER).



(D) **LPS+ *A. sativum*(300mg/kg body weight)**
High dose of *Allium sativum* was found to be relatively effective as it recovered the normal glomerular and tubular organization from degeneration of renal tubular epithelial cells and blood vessel congestion.

Treatment of animals with *A. sativum* has effect significantly at all dose levels as compared with that in LPS-induced group, with less injured area and low extent of congestion.



(E) **LPS+ standard- Dexamethasone 10mg/kg body weight.** Standard-Dexamethasone shown nearly normal renal architecture compared with the LPS treated group.

Results of the study showed that LPS significantly increase the level of NF-kB. This increase may be due to binding of LPS with TLR4 in the intestinal epithelium. This TLR4 there after activates the MyD88-dependent and TRIF-dependent pathways which form primary and secondary signals. These signals ultimately activates NF-kB, these observations are in conformation with previous report. Shin and his colleagues in 2013 noticed increase in NF-kB with LPS administration[9].

LPS also cause marked increment in the levels of TNF-α, NO, IL-6, IFN-, PGE2 and decrease in IL-10 level. These cytokines are produced for the modulation of innate immunity. These cytokines can be produced from macrophages due to LPS, infection and inflammatory stimuli. TNF-α is a key mediator of T lymphocyte and macrophage activation and forms either beneficial or detrimental effects on mammalian cells by inducing the secretion of NO and PGE2[10,11].

Increase level of nitric oxide in LPS challenged mice may be due to NO synthase 2 encoded by NOS2 gene. This increase in NO production may lead to the suppression of IL-10 and enhancement of TNF-α and IL-6[12,13].

PGE2 is primarily synthesized by Cyclooxygenase-2 (COX-2), which is also responsible for sepsis-related inflammatory symptoms. LPS also induce the over expression of COX-2 activity which might increase the level of PGE2 in the present investigation[14,15].

Allium sativum showed a potent anti-inflammatory effect against LPS induced inflammation. Previous studies shows that DADS the effective constituent of garlic decreased the LPS induced release of cytokines levels[16,17].

Decrease in NF-kB and NO may be due to terpenoids. These observations are in the agreement with previous reports. Jeong and his associates in 2002 observed that terpenoids block the degradation of IkBb protein which resulted in inhibition of NF-kB. This NF-kB thereafter inhibits IkBa phosphorylation as well as prevents the nuclear translocation of subunit p65 of NF-kB complex and also reduces the expression NO production[18,19].

Histopathological examination revealed the LPS toxicity on glomerulus, proximal tubule and distal tubule. The histological analysis of kidney tissues of the mice after LPS intoxication showed pathological event like congestion of blood vessels, focal interstitial hemorrhage and glomerular hemorrhage. Mild renal tissue distortion was also seen which suggests tissue damage while the renal architecture of control mice was normal[20]. In this work it was observed that *Allium sativum* tried to restore the normal architecture of renal tissue. This may be due to the active ingredients present in the bulb of garlic[21].

4. Conclusion

Garlic has been largely known as anti-inflammatory due to the presence of sulfur compounds. The modulation in cytokine by reducing the proinflammatory activity of NF-kB is correlated with the total bulb sulfur content present in garlic. Hence, it was deduced that garlic is enough potent to regulate the immune system.

5. Acknowledgements

The authors acknowledge the cooperation and support of Banasthali Vidyapith for providing the facility to conduct this work.

6. Conflict of Interest

The authors declare that they have no conflict of interest.

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