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# Noni fruit juice reverses the adverse effects of 3-Methyl-4-Nitrophenol in rat spleen

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Noni plant is recognized for its antioxidant properties and broad therapeutic effects. 3-Methyl-4-Nitrophenol (PNMC) poses toxic effects on various organs. This study aimed to investigate the effects of PNMC, a substance to which both animals and humans are exposed through diesel exhaust particles and widely used pesticide fenitrothion, on the number of plasma cells in the spleen and also whether Noni could alleviate PNMC-induced spleen damage in rats by assessing spleen index, plasma cell counts, and levels of TNF-α and iNOS. Eight rat groups were treated with different doses of PNMC, either alone or combined with Noni, or only Noni, or vehicle (PBS with 0.05% Tween 80, s.c.) for five days. In histological analysis, plasma cells were counted in methyl green-pyronin stained sections, and TNF-α and iNOS levels were assessed immunohistochemically. PNMC exposure significantly reduced ($P$ <0.001) plasma cell counts in the spleen, while Noni reversed this effect, particularly in the 10 mg/kg PNMC group. Noni caused a statistically insignificant decrease ($P$ <0.05) in the TNF-α index in the 1 mg/kg PNMC group. A significant rise ($P$ <0.001) in iNOS levels was observed in the 100 mg/kg PNMC+Noni group, while the 1 mg/kg PNMC+Noni combination significantly reduced ($P$ <0.001) iNOS values. These findings indicate that the inflammatory response to PNMC and its modulation by Noni are dose-dependent. Notably, Noni restored plasma cell counts in inflamed spleen to levels comparable to the control group, suggesting its protective potential in PNMC-induced spleen damage.

**Keywords**: Diesel exhaust particles, Pesticide, *Morinda citrifolia*, Plasma cell, Rat, Spleen index

As world population increases, accompanying environmental pollution becomes more prevalent and threatens the survival of humans and nature, causing more than 9 million deaths each year globally[1]. Air pollution, one of the major forms of environmental pollution, is associated with a wide range of diseases such as respiratory, cardiovascular and reproductive dysfunctions and cancer[2].

It is a more serious problem in low-income and middle-income countries[1], due to high rate of population growth, uncontrolled urbanization, industrialization, and fossil fuel combustion[2]. While there are many sources of air pollution, such as railways, airlines and other types of vehicles, it is estimated that automobiles are responsible for about 80% of today's air pollution[2].

Nitrophenols, such as 4-nitrophenol (PNP) and 3-methyl-4-nitrophenol (PNMC), are hazardous pollutants found globally in water, fog, rain, soil, and ambient fine particulate matter[3], and they affect both human and animal health[4] through oxidative stress that leads to tissue damage[5]. Diesel engines emit a complex mixture of hundreds of constituents in either gas or particle form, which is the primary source of nitrophenols. The latter form, also termed diesel exhaust particles (DEPs), consists of fine particles that are highly respirable[6]. The average amount of PNMC in DEPs is reported to be 79.1 mg/kg[7]. PNMC is also a degradation product of the fenitrothion, a widely used pesticide with high exposure potential to humans, livestock, and poultry[8]. Numerous studies have reported the adverse effects of PNMC on many systems in mammals, such as reproductive[7], urinary[9], digestive[10], endocrine[11] and cardiovascular[12] systems.

Noni (*Morinda citrifolia* L.), belonging to the Rubiaceae family, is a small evergreen tree widely distributed from Southeast Asia to Australia, Polynesia, and the Caribbean region[13,14]. In addition to antimicrobial, antifungal[13], and anticancer[15]

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properties, Noni possesses a wide range of therapeutic effects on respiratory disorders, infections, tuberculosis, diabetes[16], and immune-enhancing and hepatoprotective effects[13]. Based on chemical and nutritional analyses, Noni contains more than 200 phytochemical substances with bioactive properties[14], and the fruit is especially rich in the most valuable chemical compounds, including antioxidants[13]. From substantial *in vivo* and *in vitro* studies, Noni and Noni-derived products such as juice were understood to possess anti-inflammatory activities and the ability to scavenge free radicals and stimulate the immune system[17]. This study aimed to identify histological changes and evaluate the potential ameliorative effects of Noni fruit juice in the spleens of PNMC-treated rats by assessing spleen index, plasma cell counts, and immunohistochemical markers for tumour necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS).

## Materials and Methods

### Animals

Sexually mature male Sprague-Dawley rats were used in this study. Fifty-six rats weighing 200-280g were housed in polypropylene cages under standard conditions (22-24°C, 50% humidity, and a 12-hour light/dark cycle). To acclimate, animals had free access to tap water and standard laboratory chow for at least seven days. The Istanbul University Local Ethics Committee approved the experimental protocol for Experimental Animals (Approval No. 2013/53).

### Chemicals and experimental design

PNMC (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) containing 0.05% Tween 80 (Merck) before subcutaneous (s.c.) injection. Noni fruit juice (referred to as Noni) (99.5%) was obtained from Alnoni Ltd. (Antalya, Türkiye), and 2 mL per rat was administered by oral gavage, regardless of body weight. The rats were randomly divided into eight equal groups (n=7): one control group and seven treatment groups (Table 1). Treatments were administered daily for five consecutive days in each group.

### Spleen index (Splenic weight index)

On the sixth day of the study, the rats were individually weighed and then sacrificed using an overdose of diethyl ether anaesthesia. After excision, each spleen was weighed. The spleen index was calculated as the ratio of spleen weight (in mg) to body weight (in g) and expressed as a percentage[18].

### Plasma cell counts

The spleens were fixed in neutral buffered formalin (10%) for 24 h, routinely processed, and embedded in paraffin. To identify plasma cells, sections 4-5 µm thick were stained with methyl green-pyronin[19] (Fig. 1A). Plasma cell counts were performed using a 100-square ocular micrometre (eyepiece graticule). The cells in 100 square units of the ocular micrometre were counted at 100× magnification using an Olympus CX22 microscope. In each slide, cells from 25 randomly selected areas were counted. The

Table 1 — The experimental design in the study

| Experimental Groups | Treatment Dosages | Administration method |
|---|---|---|
| 1 | Control (PBS+%0.05 Tween 80) | s.c. injection |
| 2 | Noni (2 mL/bw) | Gavage |
| 3 | PNMC (1 mg/kg) | s.c. injection |
| 4 | PNMC (10 mg/kg) | s.c. injection |
| 5 | PNMC (100 mg/kg) | s.c. injection |
| 6 | Noni (2 mL/bw)+1 mg/kg PNMC | Gavage and s.c. injection |
| 7 | Noni (2 mL/bw)+10 mg/kg PNMC | Gavage and s.c. injection |
| 8 | Noni (2 mL/bw)+100 mg/kg PNMC | Gavage and s.c. injection |

[s.c.: subcutaneous, bw: body weight]

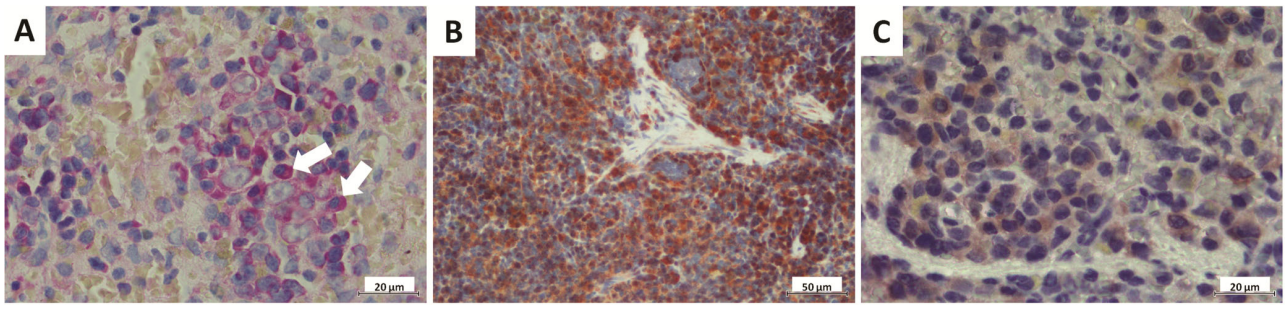


Fig. 1 — A. Plasma cells in the spleen (white arrow) (methyl green-pyronin), B. iNOS immunostaining in the spleen, C. TNF-α immunostaining in the spleen. Scale bar: A,C: 20µm, B: 50 µm

arithmetic mean of the counts was calculated, and all numerical data obtained from the 100-square ocular micrometre at 100× magnification were converted to plasma cell numbers per 1 mm² of unit area[20].

#### Immunohistochemistry

Tissue sections from paraffin blocks were collected onto positively charged slides, which were then subjected to deparaffinisation, dehydration, and microwave-assisted antigen retrieval (high power for 20 min in citrate buffer, *p*H 6.0). For TNF-α immunostaining, the slides were incubated with TNF-α antibody (Abbiotec, 2A9B, 1:1000, 1 h at 37°C) and subsequently treated with a commercial secondary antibody kit (Histostain®-Plus Bulk Kit, Invitrogen®, 85-8943), and marked with 3,3'-Diaminobenzidine (DAB) included in the kit. For iNOS immunostaining, the slides were incubated overnight at 4°C with a commercially available, ready-to-use rabbit polyclonal primary antibody for iNOS (Thermo Scientific, RB-1605-P, 1:100). They were then treated with a secondary antibody kit (Thermo Scientific). The reaction was visualised using AEC chromogen (3-amino-9-ethyl carbazole, Cat. No. TA-060-HA, Thermo Scientific). Finally, the slides were counterstained with Mayer's hematoxylin (Fig. 1B & C). Negative control slides were incubated only with antibody diluent instead of primary antibodies[21].

Two blinded observers quantified immunohistochemical TNF-α and iNOS staining at 400× magnification (Olympus BX53F microscope, Hamburg, Germany). TNF-α immunoreactivity was expressed as a percentage index (%) based on the formula (number of TNF-α positive cell nuclei/total number of cell nuclei) × 100, with 800-1,000 cells counted from 5-10 fields for each slide[22]. Immunohistochemical iNOS staining was quantified using a histological scoring system (H-scores)[21].

#### Statistical analysis

The statistical analysis of numerical data was conducted using One-way ANOVA and Duncan's multiple comparison test in SPSS 13.0 software. The results are expressed as means ± standard error (SE). Statistical significance was defined as $P \leq 0.05$.

## Results

#### Spleen index values

Spleen index values were increased in rats exposed to 1 mg of PNMC. In contrast, the values were significantly lower in the groups receiving 10 and 100 mg of PNMC compared to the 1 mg PNMC-treated group ($P < 0.05$). The group receiving Noni alone exhibited values similar to those of the control group. Overall, administering Noni to rats treated with 1, 10, and 100 mg of PNMC did not significantly differ in spleen index values. When compared to the control group, the differences in spleen index values among all Noni and PNMC-treated groups, whether combined or alone, were not statistically significant ($P > 0.05$) (Fig. 2A).

#### Plasma cell counts

In the group receiving Noni alone, the number of plasma cells was similar to that of the control group. Compared to the control group, the number of plasma cells was significantly decreased in the groups treated with 1, 10, and 100 mg of PNMC ($P < 0.001$). However, this decrease was not dose-dependent. When Noni was administered to rats receiving 1, 10, and 100 mg of PNMC, the number of plasma cells increased, with statistically significant differences observed between the groups receiving PNMC alone and those receiving the combination of PNMC and Noni ($P < 0.001$). Notably, the number of plasma cells in the group receiving combined Noni and 10 mg of PNMC was close to that of the control group ($P > 0.05$) (Fig. 2B).

#### TNF-α values

The TNF-α index in the group receiving Noni alone was similar to that in the control group. Administration of 1, 10, and 100 mg/kg PNMC caused an increase in TNF-α indices compared to the control group, but the differences among the groups were nonsignificant ($P > 0.05$) and dose-independent. Noni caused a statistically insignificant decrease in the TNF-α index in the group treated with 1 mg/kg PNMC. No statistically significant differences in TNF-α indices were observed between the groups receiving PNMC and those receiving PNMC combined with Noni. There were a statistically significant difference between the group receiving Noni alone compared to the groups receiving 10 mg/kg PNMC and group receiving the combination of PNMC (10 mg/kg) and Noni ($P < 0.01$) (Fig. 2C).

#### iNOS values

iNOS values, quantified by H-score, in the groups receiving Noni, 1, and 10 mg/kg PNMC alone were close to those of the control group. However, a significant increase in iNOS values was found in the

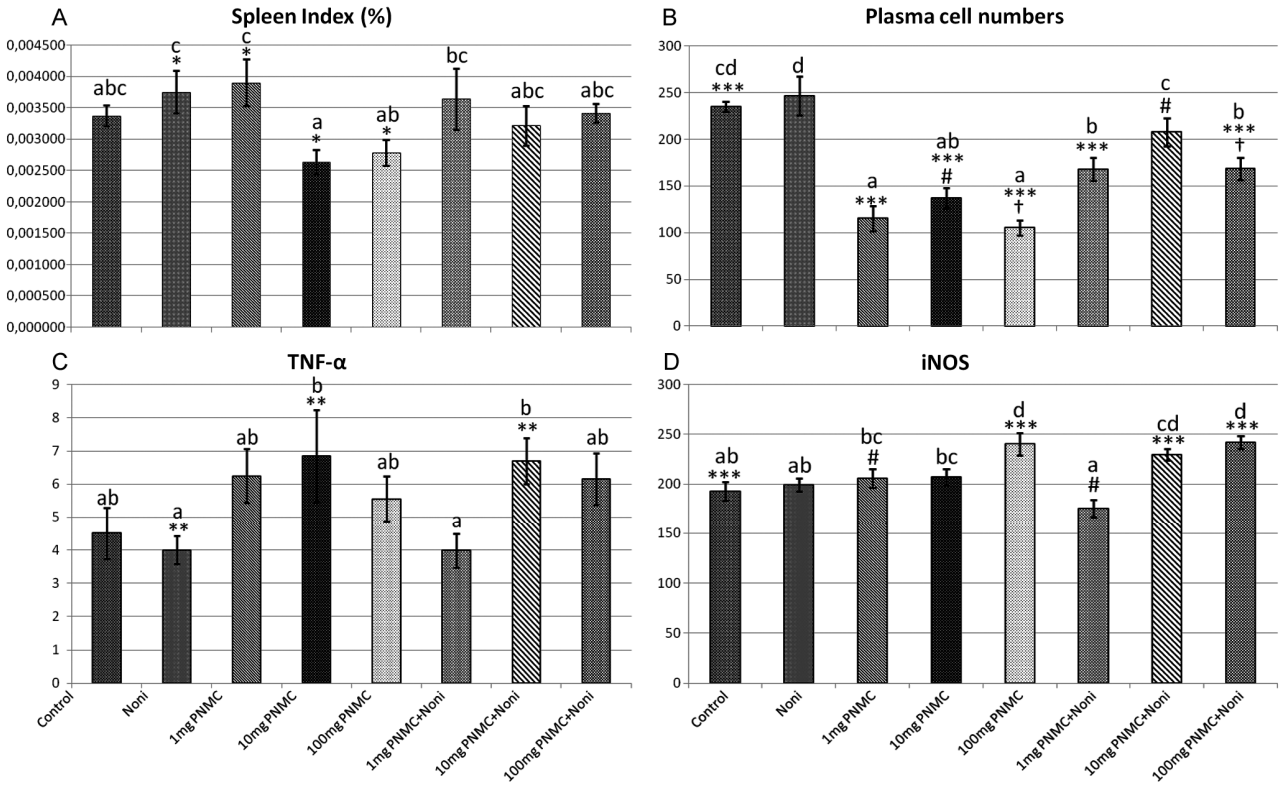


Fig. 2 — (A) Spleen index, (B) Plasma cell numbers, (C) TNF-α index (%), (D) iNOS H-score levels. Differences between values that do not share a common letter are statistically significant ($P$ <0.001 in Fig. 1A, B, D; $P$ <0.05 in Fig. 1C)

group treated with 100 mg/kg PNMC compared to the control group ($P$ <0.001). A considerable increase was also present in the group receiving the combination of PNMC (100 mg/kg) and Noni ($P$ <0.001). A significant decrease in iNOS values was observed in the group treated with 1 mg/kg PNMC and Noni compared to the group receiving 1 mg/kg PNMC alone ($P$ <0.001) (Fig. 2D).

## Discussion

The present study investigated the potential ameliorative effects of Noni on the spleen of PNMC-treated rats based on the analysis of spleen index and plasma cell counts in methyl green-pyronin-stained sections of spleen tissue. Additionally, TNF-α and iNOS were assessed as markers of inflammation that PNMC may induce in immunohistochemically stained tissue sections. Sexually mature male Sprague-Dawley rats were used in this study. In our study, in addition to Noni application alone, PNMC doses of 1, 10, 100 mg/kg were applied to the experimental groups either alone or in combination with Noni. It was reported that the amount of potassium in Noni Juice to be as 56.3 mEq/L[23], and consuming Noni fruit juice above a certain amount could cause hyperkalemia. In the light of the literature data, Noni administration in our study was carried out via gavage at a dose of 2 mL/bw. There are studies stating that doses higher than the Noni dose used in our study, causes adverse effects[24,25]. For this reason, Noni was not administered at doses higher than 2 mL/bw in our study.

The spleen index values in the group receiving Noni alone were similar to those of the control group. Consistent with this finding, previous studies have reported that Noni has no effect on spleen weights in mice[26] and rats[27], as well as on the body weights of rats[28]. While some studies have reported that fenitrothion causes significant decreases in spleen/body and thymus/body weight ratios in mice[29] and rats[30,31], PNMC has shown no significant effect on spleen and body weight[8,9]. This study observed a slight decrease in the spleen index in the groups receiving 10 and 100 mg/kg of PNMC compared to the control. In the groups treated with combined PNMC and Noni, the spleen index values for those receiving 10 mg/kg and 100 mg/kg of PNMC+Noni were closer to those of the control

group, but the differences were not statistically significant.

Although several previous reports indicate the effects of fenitrothion and PNMC on immune cell populations in the spleen[31-33], studies on the relationship between PNMC and the number of plasma cells in the spleen are lacking. A study in mice elucidated that Noni fruit extract did not cause any changes in the T and B lymphocyte subpopulations in the spleen[34]. In contrast, significant splenocyte proliferative effects were observed with water and hydroalcoholic extracts of Noni, indicating that these extracts had a mitogenic effect on lymphocytes[35]. Moreover, Hong *et al.*[26] reported that the water extract of Noni treated with pectic enzyme Mc-eWE stimulates CD8+ T cell-mediated adaptive immune responses by inducing the secretion of cytokines specific to T-cells and B-cells rather than by increasing the populations of these cells. The current study found a decrease in the number of plasma cells in the spleens of PNMC-treated rats. Plasma cells develop from B lymphocytes[36] residing in the lymph nodes and spleen, migrating into inflamed tissues where they persist[37,38]. Thus, the decreased number of plasma cells in the spleen of PNMC-treated rats may result from their migration into inflamed tissues caused by PNMC. When Noni and PNMC were administered together, the number of plasma cells increased, approaching that of the control group. Numerous studies have shown that Noni exerts antioxidant and anti-inflammatory effects, attenuating inflammation[13,21,39,40]. Consequently, the migration of plasma cells from the spleen to the inflamed tissues may be decreased in rats treated with the combination of Noni and PNMC.

Previous studies have demonstrated an increase in inflammation markers and oxidative stress parameters in various organs of animals, including mice[7], rats[21] and chickens[41], exposed to PNMC. TNF-α and other cytokines support plasma cell survival and act as modulators of inflammation, indicating that inflamed tissues may create optimal conditions for plasma cells[42]. *In vitro* incubation of splenocytes with Noni significantly increased TNF-α production in isolated rat splenocytes; however, *in vivo* treatment with Noni did not considerably affect TNF-α production[28]. This finding aligns with the observation that the TNF-α index of the group receiving Noni alone was similar to that of the control group. Hong *et al.*[26] reported that Mc-eWE-treated Noni upregulated the mRNA expression of TNF-α and significantly increased TNF-α production in mouse splenocytes dose-dependently.

Oral exposure to fenitrothion at a dose of 20 mg/kg body weight per day for 30 days caused an increase in serum TNF-α levels in rats[43]. However, the production of TNF-α was not affected by fenitrothion at concentrations that significantly inhibited the proliferation of peripheral blood mononuclear cells and T cell-derived cytokine production[44]. In this study, Noni caused a statistically insignificant decrease in the TNF-α index in the 1 mg/kg PNMC-treated group. At the same time, other doses of combined Noni and PNMC did not result in statistically significant differences in TNF-α indices. The variation in TNF-α results across different studies may be attributed to differences in application durations, doses, routes, and the tissues investigated[43,44]. Additionally, despite PNMC being a metabolite of fenitrothion, the different chemical properties of these substances may lead to distinct results.

In the study by Yang *et al.*[32], PNMC caused significant increases in hydroxyl free radical (OH) content and marked decreases in superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels in splenocytes. Increased production of hydroxyl radicals and hydrogen peroxide, as well as lipid peroxidation and decreased levels of glutathione, SOD, and GSH-Px activities, were also reported in the testes of mice after exposure to PNMC[7]. Reactive nitrogen species such as nitric oxide (NO) are elevated due to oxidative stress, and pesticide toxicity notably induces the generation of iNOS. As a biomarker of inflammation and oxidative stress, iNOS is commonly used in toxicity studies, including those related to pesticide toxicity in various organisms[45].

Pratap *et al.*[28] demonstrated that Noni treatment significantly increased NO production in the splenocytes of rats. Previous research showed that administration of PNMC at doses of 1, 10, and 100 mg/kg led to significant increases in iNOS levels in a dose-dependent manner in the testes of rats, and elevated iNOS levels were significantly reduced following Noni application[21]. However, there is no available data for comparison on iNOS levels in spleen injury induced by PNMC. In the current study, iNOS levels were significantly increased in the groups

receiving 100 mg/kg PNMC, alone or combined with Noni, compared to the control. In contrast, doses of 1 and 10 mg/kg PNMC did not lead to any increase in iNOS levels. Noni caused a significant decrease in the combined 1 mg/kg PNMC and Noni group compared to the group receiving 1 mg/kg PNMC alone. It can be inferred that the same dose of PNMC may produce different effects on iNOS levels in various tissues.

## Conclusion

The results of our study revealed that the application of Noni, which has antioxidant properties, ensures that plasma cell numbers in animals exposed to the negative effects of PNMC are similar to the control group and shows a protective effect against the damage caused by PNMC. It has been determined that Noni didn't cause a significant difference in inflammation marker TNF-α results and causes a decrease in inflammation marker iNOS results only in 1 mg/kg PNMC and Noni combination group. According to these results, it has been shown that oral administration of Noni has positive effects on plasma cell numbers in the spleen of rats which exposed to various doses of PNMC and on iNOS levels in the spleen of 1 mg/kg PNMC administered rats. While numerous studies have explored the effects of PNMC on various organs, there remains a scarcity of information regarding its impact on plasma cells in the spleen. This study is the first to investigate the effects of PNMC on the number of plasma cells in the spleen and to assess the extent to which Noni, known for its antioxidant and anti-inflammatory properties, can modulate changes induced by PNMC.

## Ethical statement

The Istanbul University Local Ethics Committee approved the experimental protocol for Experimental Animals (Approval No. 2013/53).

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## Conflict of interest

The authors declare no competing interests.

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