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Research Article

IN VITRO SCREENING OF *VITEX NEGUNDO* LEAF EXTRACT FOR ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY AND ITS *IN VIVO* EFFECTS ON OXIDATIVE STRESS IN LPS-INDUCED LUNG INJURY

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ABSTRACT: This study investigated the *in vitro* antioxidant, antimicrobial activity and in vivo antioxidant potential of *Vitex negundo* leaf extract (VNLE) in LPS-induced lung injury in mice. VNLE at doses of 150 and 300 mg/kg body weight and dexamethasone at the dose of 5 mg/kg body weight were given for 7 days before LPS administration. In vitro antioxidant activity was evaluated using DPPH and nitric oxide scavenging assays, demonstrating the neutralization of radicals (DPPH: 86.91% inhibition; IC50 = 49.04 µg/mL; nitric oxide: 93.33% inhibition; IC50 = 63.13 µg/mL). In addition, total phenolic content (TPC) and total flavonoid content (TFC) in VNLE were also quantified, showing TPC 56.22 ± 4.88 mg QE/g and TFC 196.3 ± 1.78 mg QE/g. In the disc diffusion assay, VNLE at a concentration of 100 mg/mL produced a 6 mm zone of inhibition against *E. coli*. Furthermore, the *in vivo* evaluation of antioxidant effect of VNLE in serum revealed that LPS treatment significantly reduced glutathione and catalase activities, while VNLE pretreatment notably restored these levels, especially at the higher dose of 300 mg/kg. Additionally, malondialdehyde level, indicative of oxidative stress, was modulated by VNLE pretreatment. Overall, the findings suggest that VNLE possesses notable antioxidant effect and highlighting its potential in managing oxidative stress induced by LPS in lung injury model of mice.

Keywords: *Vitex negundo* leaf extract, LPS, ALI, Free radical scavenger, Oxidative stress, Antibacterial activity.

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common life-threatening lung diseases with high mortality rates [1]. ALI is driven by oxidative stress, with reactive oxygen species (ROS) like superoxide, hydrogen peroxide, and hydroxyl radicals playing a central role in lung damage [2]. These ROS, along with reactive nitrogen species, damage DNA, lipids, and proteins, contributing to inflammation and tissue destruction [3]. Neutrophils and other lung cells are major sources of ROS, and experimental models and clinical studies show elevated oxidative stress markers in ALI/ARDS cases [2]. Lipopolysaccharide (LPS), a main component of Gram-negative bacteria, has been linked to an increased risk of ALI, which can be triggered by LPS or immune complexes that activate neutrophils and macrophages, leading to liberate of oxidants and subsequent lung damage [4]. *Vitex negundo* Linn is a remedial plant of *Verbenaceae* family, known as punjgusht, nirgundi, or five leaved chastes tree [5]. This medicinal plant is reported with anti-inflammatory, analgesic, anti-oxidant, immunomodulatory, cytotoxic, insecticidal, anti-convulsive; anti-microbial, anti-neoplastic and anti-allergic properties [6,7]. Based on the plant traditional usage as medicine for hyperactive respiratory conditions including bronchitis, cough, and asthma, an extract from leaves of the *Vitex negundo* plant has been shown to be beneficial in experimental model of asthma [8]. However, there is a lack of available literature demonstrating the antioxidant effects of VNLE in LPS-induced lung injury. In order to assess in vitro

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anti-oxidant property of VNLE as well as its effects on in vivo after LPS-induced acute lung damage in mice, the current study has been designed.

MATERIALS AND METHODS

Animals

Healthy adult male Albino mice (25-30 g) were procured from the Laboratory Animal Resource (LAR) Section, ICAR-IVRI, Izatnagar, (U.P.) India. Mice were housed in the polypropylene cages under 12-12 h dark light cycle with freely accessible balanced standard feed and water. Animals were kept for an acclimatization period of 07 days before the conduction of experiments. Every protocol used complied with the guidelines set forth by the Institutional Animal Ethic Committee (IAEC proposal no. IAEC/06.10.2023/S8) of ICAR-IVRI, Izatnagar, Uttar Pradesh.

Experimental design

Five groups of mice were selected at random, with a minimum of six mice in each group. Control mice (Group-I) were received phosphate buffered saline, intranasally and gum acacia as vehicle for 07 days via oral route (PO). Pretreatment of VNLE in Group IV (150 mg/kg body weight), Group V (300 mg/kg body weight) and dexamethasone in Group III (5 mg/kg body weight) were administered for 07 days orally (P.O.) in mice. On 07th day, lipopolysaccharide (LPS) was given by intranasal (I/N) route in Group II, III, IV and V under suitable anesthesia. Mice were sacrificed after 24 hrs of LPS administration using suitable anesthesia [9]. Blood was collected by cardiac prick from mice of different groups in serum separator tube (SST) and allowed to clot for two hours at ambient temperature. Serum was separated by centrifugation at 4000 rpm for 10 min and kept at -80℃ for further use. Total Protein concentration was determined in serum by bradford assay.

Preparation of extract

Vitex negundo leaves were obtained from the ICAR-IVRI campus in Izatnagar, Bareilly, UP, India. The leaves were identified and authenticated by the Botany Department of Bareilly College, Uttar Pradesh. After washing of leaves under running water, they were then washed with distilled water to get removal of dust and other contaminants. After cleaning, the leaves were shade-dried for 10 days at room temperature. Once dried, they were crushed and immersed in 70% ethanol (1g/10ml) for three days, with frequent shaking. After straining the mixture, a rotary evaporator was employed to concentrate the ethanolic extract at 40°C. The semi-solid residue obtained was then dried in a vacuum desiccator and lyophilized (freeze-dried) to achieve a fine powdered form.

Total phenolic concentration (TPC)

The TPC was computed using the spectrophotometric technique, as reported by Stankovic & co-workers [10]. To put it briefly, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water, 0.5 mL of extract solution (1 mg/mL), and 2.5 mL 7.5% $NaHCO_3$ were mixed to prepare reaction mixture. After 15 min of incubation at 45 °C, absorbance of samples was measured at 765 nm. The samples were arranged in triplicates, and the mean absorbance value was determined. Instead of using the extract solution, ethanol was used for preparing blank concurrently. The calibration line was exhibited and the process was repeated for the quercetin.

Total flavonoid content (TFC)

TFC of VNLE was concluded using aluminum chloride ($AlCl_3$) assay. In a nutshell, a test vial containing 60 µL of 5% (w/v) $NaNO_2$ was filled with 10 mg of extract and one milliliter of distilled water. Five minutes later a 10% (w/v) $AlCl_3$ solution (60 µL) was poured. 1M NaOH (400 µL) was added after 6 minutes, bringing the total volume 2 ml using distilled water. The solution was mixed thoroughly, and the activity absorbance was noted at 510 nm against a reagent blank. A QE standard curve was employed to calculate concentrations. Milligrams of quercetin per gram of extract was the measure used to express the mean total flavonoid levels (n = 3).

Assessment of *in vitro* free radical neutralizing activity

DPPH neutralizing assay

The Blois protocol [11] was performed to assess the DPPH radicals neutralizing ability of VNLE and ascorbic acid standard. A freshly prepared DPPH solution of 0.1 millimolar in ethanol, which appears violet/purple, was used. In the experiment, 1.6 milliliters of extract or standard at different concentrations (6.25–200 µg/ml) was combined with 2.4 milliliters of DPPH mixture. Antioxidants cause the purple tint to disappear, showing proton donation. Absorbance was assessed after 30 min at the wavelength of 517 nanometer using an ELISA reader, with a blank containing no test compounds. The percentage inhibition activity was calculated using the equation:

DPPH neutralizing capacity = $(A_0 - A_1)/A_0) \times 100$

Where A_0 is optical density of control and A_1 is optical density of extract/standard (ascorbic acid). Inhibition % was then plotted against the concentration, and IC50 value was calculated from the curve.

Nitric oxide (NO) neutralizing assay

NO produced from sodium nitroprusside (SNP) in ethanol at physiological pH was measured using method of Marcocci and co-workers [12], with nitrite ions estimated by Griess reagent. Sodium nitroprusside (10 mM) of 2 ml, phosphate buffer saline (pH 7.4) of 0.5 ml, and 0.5 milliliter of standard ascorbic acid (15.62–500 µg/ml) constituted the 3 ml reaction mixture. It was incubated for 150 minutes at 25°C. Following incubation, 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) was added to 0.5 ml of mixture, and the mixture was subjected to incubation for 5 minutes to allow for diazotization. Subsequently, one milliliter of naphthyl ethylene diamine dihydrochloride (NEDD) (0.1% w/v) was infused on to the samples, and they were left to stand at 25°C for 30 minutes. The nitrite concentration was calculated at 546 nm, using absorbance of a standard nitrite solution as the control, ascorbic acid (as standard) and blank containing buffer. The percentage inhibition was determined.

Percent scavenging activity = $\{(A_{control} - A_{test} \text{ or } As_{td})/ A_{control}\} \times 100$

Where $A_{control}$ is control optical density and A_{test} or A_{std} is test or standard optical density, respectively.

Antibacterial study

The antibacterial activity of the *Vitex negundo* extract with different concentration was studied against gram-negative bacteria, *Escherichia coli* using disc diffusion method [13]. Sterile discs (HiMedia) were infused with 30 µl of 12.5 mg/ ml, 25 mg/ ml, 50 mg/ ml and 100 mg/ ml solutions of the crude extract and then let them dry until the evaporation of the solvent. The density of the test culture was adjusted with 0.5 McFarland tubes. Further, using a sterile cotton swab the density-adjusted culture was spread on Muller Hinton agar (MHA) and left for drying. The discs containing the extracts were applied over the culture-inoculated MHA plates and kept at 37° for 24 hrs. Commercially available antibacterial discs (HiMedia) such as kanamycin (30 µg), imipenem (10 µg) cefotaxime/clavulanic acid (30/10µg), amikacin (30 µg), erythromycin(15 µg) and Ofloxacin (5µg) were served as a control. The extract-infused discs and commercial antibiotics discs were incubated, and the zone of inhibition was determined in millimeters.

Estimation of anti-oxidative enzymes

The protocol of Madesh and Balasubramanian [14] was adopted to calculate the activity of superoxide dismutase (SOD). The chemical mixture was kept for five minutes incubation. It includes tissue homogenate, 1.25 mM MTT, 100 µM pyrogallol, and 0.65 mL PBS (pH 7.4). Dimethyl sulfoxide was added to stop the reaction, and the activity absorbance was noted at 570 nm. SOD units were utilized to express the results. The GSH level was investigated with use of Sedlak and Lindsay [15] protocol. After combining the tissue homogenate sample, 50% trichloroacetic acid, and distilled water, the mixture was incubated for 15 min at 37 ℃ before it was spun for fifty minutes at 3000 revolutions per minute. 0.01M DTNB (5, 52-dithiobis-2-nitrobenzoic acid) and 0.4 ml of supernatant were mixed with 1 M Tris buffer (pH 8.9). The optical density was measured swiftly (within 5 minutes) at 412 nm. The Aebi [16] method was utilized to calculate catalase activity. The tissue sample was added to 50 mM phosphate buffer (pH 7.0). Following the addition of 30 mM hydrogen peroxide (H_2O_2), the absorbance at 240 nm was measured in every 15 seconds for 1 minute. Catalase activity was calculated using the millimolar (mM) coefficient of extinction of H_2O_2. The results were derived with chromophores 13000/M/cm molar extinction coefficient. Using the Buege and Aust [17] method, the amount of malondialdehyde (MDA) indicates lipid peroxidation. Tissue homogenates were blended with 1:1:1 TBA-TCA-HCl (Thiobarbituric acid-Trichloroacetic acid-Hydrochloric acid) reagent (TBA 0.37%, 15% TCA and 0.25N HCl). Following that, the mixture was placed in a boiling water bath over fifteen minutes. The clear supernatant absorbance was then measured at 535 nm with respect to the blank. Using malonaldehyde's molar absorptivity of 1.56×10^5 $M^{-1}cm^{-1}$, the concentration was determined.

Statistical analysis

With n denoting the number of mice, the results are shown as mean ± SEM. To analyze the data, GraphPad Prism Version 8.0 was utilized. Tukey's post-hoc test and one-way ANOVA were used for multiple comparisons. At $p < 0.05$, statistical significance was determined.

RESULTS AND DISCUSSION

Concentration of total phenolics

Both edible, inedible plants, and plant components, frequently contain phenolic substances [18]. They

have been linked to a number of biological perks, including antioxidant activity. Because of their redox characteristics, TPC may directly contribute to the antioxidative effect. These characteristics may be essential for dissolving peroxides, reducing single-molecule and triplet oxygen, and absorption and neutralization of reactive oxygen species [19]. Additionally, it was previously established that phenol, flavonoids, terpenoids, anthraquinones, polysaccharides, and steroids are present in *Vitex negundo* [20]. In consistent of above, the VNLE showed a TPC of 56.22 ± 4.88mg QE/g of extract designated as quercetin equivalent or QE (equation of standard curve: y = 0.002x + 0.1659, r^2= 0.991) as mg QE/g of extract. In accordance to our findings, Saklani and colleagues [21] have found that the TPC in *Vitex negundo* leaves was 89.71 mg GAE/g extract (Table 1).

Table 1. Secondary metabolite contents in *Vitex negundo* leaves

Parameter analyzed	*Vitex negundo*
Total Phenolic Content (mg QE/g dry weight of extract)	56.22 ± 4.88
Total Flavonoid Content (mg QE/g dry weight of extract)	196.3 ± 1.78

Concentration of total flavonoid

The most significant natural phenolics are flavonoids, which possess a wide range of biological and chemical characteristics, including the ability to destruct reactive oxygen species [22]. There is indication to sustain the idea that consuming up to 1.0 g of polyphenolic chemicals per day, derived from vegetables and fruits, can reduce development of cancer and mutagenesis in human [21]. TFC was determined from the graphical formula (y = 0.0003x + 0.0413, r^2= 0.992), represented as a quercetin equivalent (QE). VNLE exhibited TFC as 196.3 ± 1.78mg QE/g of extract. Consistent with our findings, a previous study by Lakshmanashetty & coworkers [23] reported that TFC in *Vitex negundo* extract was 166.67 ± 9.14 mg catechin per gram of extract. (Table 1).

DPPH scavenging activity of VNLE

The ethanolic DPPH solution was employed to estimate the antioxidant property of the leaf extract of *Vitex negundo*. When DPPH is reduced in an ethanol solution containing coloured free radicals, either by hydrogen or electron donation, the stable nitrogen

Table 2. Zone of inhibition (mm) of ethanolic *Vitex negundo* leaf extract (12.5-100 mg/ml) and different antibiotics against *E. coli* pathogen

Sl. No.	Drug/ Extracts	Zone of Inhibition (in mm)
1)	VNLE 100 mg/ml	6
2)	VNLE 50 mg/ml	3
3)	VNLE 25 mg/ml	4
4)	VNLE 12.5 mg/ml	4
5)	Kanamycin 30 µg	9
6)	Imipenem 10 µg	15
7)	Cefotaxime/ Clavulanic acid 30/10 µg	11
8)	Amikacin 30µg	9
9)	Erythromycin 15 µg	5
10)	Ofloxacin 5 µg	13

cantered free radicals colour transforms from purple to yellow [24]. This technique is often used to calculate antioxidants' capacity to eliminate radicals that are unstable. *Vitex negundo* extract showed noteworthy DPPH neutralizing effect which has increased with concentration (Fig.1). The % inhibition of extract was found 86.91% (IC50 = 49.04µg/mL). In addition, we compared neutralizing effect of our extract with ascorbic acid that showed % inhibition 92.36% (IC50= 6.95µg/mL). *Vitex negundo* extract and Ascorbic acid exhibited a comparable antioxidant activity. The antioxidant effect of extract is believed to result from its ability to eliminate radicals of DPPH due to its capacity to donate hydrogen atoms as reported in previous study [21].

Nitric Scavenging activity of VNLE

Nitric oxide, a key factor in inflammation, can react with macromolecules in an in vitro nitric oxide scavenging assay, and potentially triggering many inflammatory disorders [25]. The extract of *Vitex negundo* exhibited 93.33% NO inhibiting effect, with an IC50 value of 63.13 µg/mL. In comparison, ascorbic acid showed a higher % inhibition that was 97.50%, with a lower IC50 value of 30.30 µg/mL (Fig 2). According to our study, the extract showed nitric oxide scavenging efficacy comparable to standard. Our extract IC50 value exceeded the standard value, which indicates a reduced activity of extract.

Antibacterial activity of VNLE

The primary risk factors for acute lung injury and sepsis are infections caused by gram-negative bacteria, which release lipopolysaccharides [26]. LPS acts as a potent trigger for inflammation and oxidative stress. The tested antibiotics in this study (kanamycin, imipenem, cefotaxime/clavulanic acid, amikacin, erythromycin and Ofloxacin) showed zones of inhibition (ZOI) against the tested gram-negative E. coli pathogen, indicating their effectiveness (Table 2). Research linked to screens plant extracts for their antibacterial characteristics indicates that plants are possible resource for future anti-infective medications [27]. Furthermore, the VNLE tested at various concentrations (12.5–100 mg/mL) produced a ZOI as shown in Table 2. The zone of inhibition at 100 mg/ml of extract was found 6 mm suggested the tested bacterial strain less sensitive to the extract. One of the previous studies reported a zone of inhibition (ZOI) of 13.1 mm against E. coli at a concentration of 100 mg/mL for methanolic *Vitex negundo* extract [28]. In contrast to our findings another study demonstrated the antibacterial activity of methanolic *Vitex negundo* extract, reporting zone of inhibition (ZOI) values of 23 mm, 22.1 mm, 19.6 mm, 18.5 mm, and 17.8 mm against *E. coli*, *Staphylococcus, Pseudomonas, Enterobacter,* and *Salmonella typhimurium,* respectively [29].

Effect of VNLE on serum enzymatic and non-enzymatic anti-oxidant in LPS-induced ALI

The inherent defensive systems of the cell that counteract oxidative stress brought on by ROS are called endogenous or enzymatic antioxidants [30]. The important antioxidants are considered to include superoxide dismutase (SOD), reduced glutathione (GSH) and, catalase (CAT) [31]. SOD catalyses conversion of superoxide radicals into molecular oxygen and H2O2, whereas CAT simultaneously breaks down H2O2 into H2O and oxygen [32]. As shown in fig. 3, a significant ($p<0.05$) decrease in SOD activity was noted in LPS induced injury group (1.83 ± 0.176 units/mg

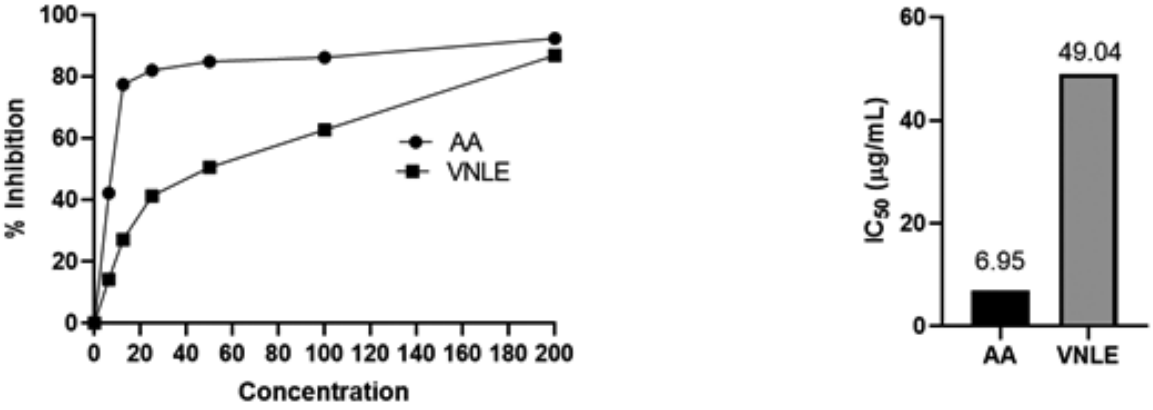

Fig. 1. Estimation of A) DPPH free radical neutralizing activity of standard Ascorbic acid (AA) and ethanolic *Vitex negundo* leaves extract (VNLE); B) IC50 (µg/mL) of AA and VNLE.

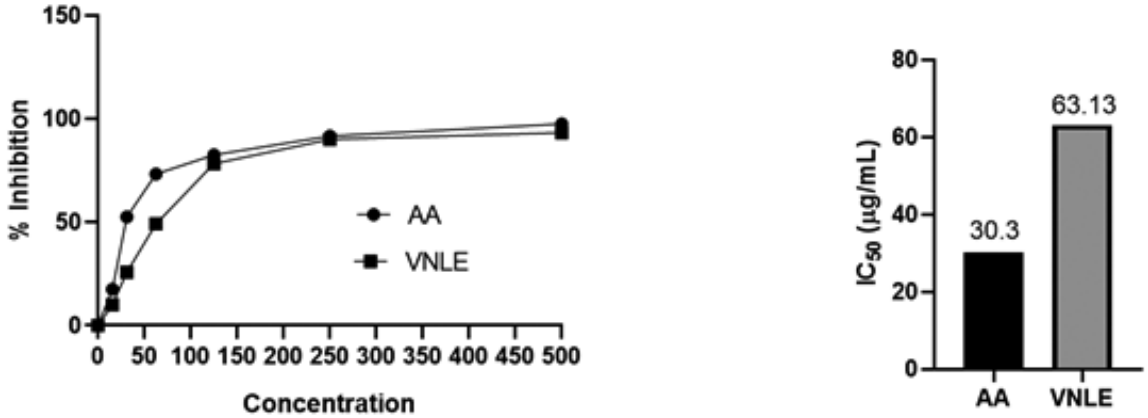

Fig. 2. Estimation of A) Nitric oxide radical neutralizing activity of standard Ascorbic acid (AA) and ethanolic *Vitex negundo* leaves extract (VNLE); B) IC50 (µg/mL) of AA and VNLE.

of protein; n=6) as compared to the untreated control (4.732 ± 0.329 units/mg of proteins; n=6). SOD activity increased with the pretreatment of *Vitex negundo* extract, particularly at dose of 300 mg/kg body weight (3.31 ± 0.69 units/mg of protein; n = 6), compared to LPS-injured mice, although the increase was not statistically significant. Furthermore, when comparing the 150 mg/kg extract pretreatment group (2.14 ± 0.18 units/mg of protein; n = 6) to the LPS-injured mice, no appreciable difference was noticed in SOD activity. Further, Data revealed that LPS administration in significantly reduced the catalase (21.40 ± 6.23 mmol H2O2 utilized/min/mg of proteins; n=6; $p<0.05$) and reduced glutathione level (0.238 ± 0.06 fold change; n=6; $p<0.001$) in respect to control (catalase 53.58 ± 9.85 mmol H2O2 utilized/min/mg of proteins; n=6; reduced glutathione 1.00±0.20 fold change; n=6). Pretreatment with *Vitex negundo* extract at 300 mg/kg *bwt* significantly increased catalase (54.16 ± 8.51 mmol H2O2 utilized/min/mg of proteins; n=6; $p<0.05$) and reduced glutathione levels (0.711±0.05 fold change; n=6; $p<0.05$). However, these values were not altered with a 150 mg/kg dose of extract compared to LPS group (catalase 47.68 ± 8.87 mmol H2O2 utilized/min/mg of proteins; n=6; reduced glutathione 0.473±0.04 fold change; n=6). Further, no statistical difference was observed in dexamethasone co-administered group with LPS in comparison with other treatment groups for SOD (2.538 ± 0.27 units/mg of protein; n = 6), CAT (26.42 ± 3.05 mmol H2O2 utilized/min/mg of proteins; n=6) and GSH (0.460±0.02 fold change; n=6), respectively. Consistent with our present findings, a previous study which reported that *Vitex negundo* extract of leaves exhibited antioxidant effects by increasing levels of SOD, catalase, and reduced glutathione in complete freund's adjuvant-induced arthritic rats [33]. Furthermore, *Vitex negundo* extract rich phytochemical composition, capacity to chelate metals, decrease oxidants, neutralize radicals that are harmful, and reduction peroxidation of lipids all contribute to its strong antioxidant capasity [21].

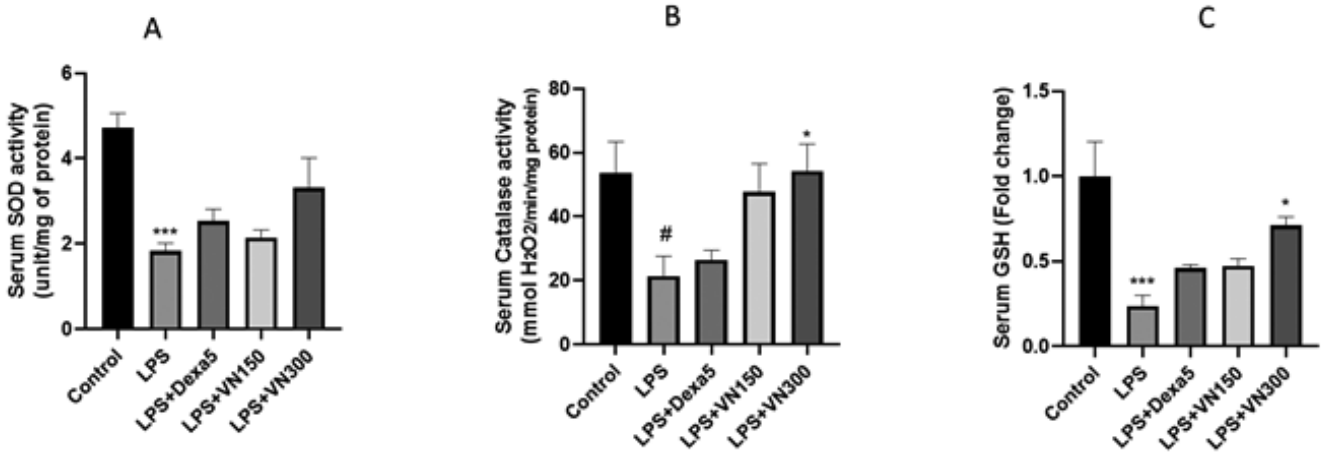

Fig. 3. Pre-treatment effect of VNLE on serum SOD (A), Catalase (B) Reduced glutathione (GSH) (C) in LPS induced ALI. [One-way ANOVA followed by Tukey's multiple comparison test. ***$p < 0.001$ & #$p < 0.05$ in comparison with Control; *$p < 0.05$ in comparison with LPS; n=6].

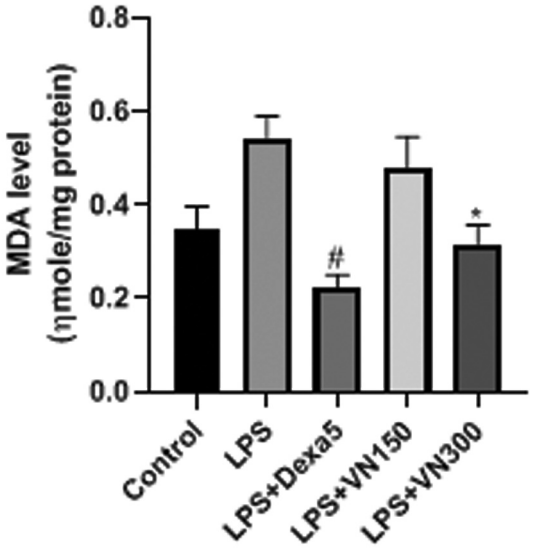

Fig. 4. Pre-treatment effect of VNLE on serum MDA in LPS induced ALI. [Data analyzed by One-way ANOVA followed by Tukey's multiple comparison test. #$p < 0.001$ & *$p < 0.05$ in comparison with LPS; n=6]

Effect of VNLE on serum malondialdehyde in LPS-induced ALI

Malondialdehyde is among the best and most precise indicators of oxidative damage [34]. Level

of malondialdehyde is generally used to assess lipid peroxidation. Figure 4 depicts, compared to the control group (0.349 ± 0.05 nmol/mg protein of serum; n = 6), the MDA level was higher in the LPS-induced injury group (0.543 ± 0.05 nmol/mg protein of serum; n = 6). Nevertheless, no significant changes were observed in MDA level with the pretreatment of dose 150 mg/kg BWT (0.479 ± 0.06 nmol/mg protein of serum; n=6). Further, pretreatment with 300 mg/kg BWT of the extract significantly reduced MDA level (0.315 ± 0.04 nmol/mg protein of serum; n=6) compared to LPS treated mice. Consistent with our current results, prior research has shown that *Vitex negundo* ethanolic extract considerably decreased MDA level in thioacetamide-induced nephrotoxicity [35] and ethanol-induced brain oxidative stress in rats [36].

CONCLUSION

In conclusion, the extract showed free radical arresting effect, particularly for nitric oxide and DPPH radicals. Additionally, the extract showed efficacy in reducing stress caused by oxidative damage by restoring antioxidant levels in mice with acute lung injury generated by lipopolysaccharide (LPS).

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