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Aqueous extract of *Ocimum gratissimum* Linn and ascorbic acid ameliorate nicotine-induced cellular damage in murine peritoneal macrophage

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ABSTRACT

Objective: To test the *in vitro* protective role of aqueous extract of *Ocimum gratissimum* Linn. *(O. gratissimum)* and ascorbic acid against nicotine-induced murine peritoneal macrophage. **Methods:** Peritoneal macrophages from mice were treated with nicotine (10 mM), nicotine (10 mM) with aqueous extract of *O. gratissimum* (1 to 25 μg/mL), and nicotine (10 mM) with ascorbic acid (0.01 mM) for 12 h in cell culture media, while the control group was treated with culture media. Levels of free radical generation, lipid peroxidation, protein carbonyls, oxidized glutathione levels and DNA damage were observed and compared. **Results:** Phytochemical analysis of aqueous extract has shown high amount of phenolics and flavonoids compound present in it. The significantly increased free radical generation, lipid peroxidation, protein carbonyls, oxidized glutathione levels and DNA damage were observed in nicotine-treated group as compared to the control group; those were significantly reduced in aqueous extract of *O. gratissimum* and ascorbic acid supplemented groups. Moreover, significantly reduced antioxidant status in nicotine exposed murine peritoneal macrophage was effectively ameliorated by these two products. Among the different concentration of aqueous extract of *O. gratissimum*, the maximum protective effect was observed at 10 μg/mL which does not produce any significant change in the normal cell. **Conclusions:** These findings suggest the potential use and beneficial role of *O. gratissimum* as a modulator of nicotine-induced cellular damage in murine peritoneal macrophage.

1. Introduction

Nicotine, an alkaloid, composed of a pyridine and a pyrrolidine ring, is found in the plant kingdom throughout a wide range of families. Tobacco smoking, cigarette smoking, chewing tobacco, and also nicotine replacement therapies are the main important sources of exposure to nicotine, since nicotine is the main active ingredient. Nicotine has been recognized to result in oxidative stress by inducing the generation of reactive oxygen species (ROS)[1,2]. The immune cells use ROS for carrying out their normal functions but an excess amount of ROS can attack cellular components and lead to cell damage. Previous in *vivo* studies from our laboratory have shown that, nicotine administration results in the imbalance of prooxidant/antioxidant status in the liver, kidney, heart, lung and spleen of male Wister rats[3]. I*n vitro* experiments with mice peritoneal macrophages also established that nicotine dose dependently generated superoxide radical, damaged the lipid & protein, and diminished the antioxidant status in peritoneal macrophages[4].

Medicinal plants are widely employed in folk medicine, mainly in communities with inadequate conditions of public health. Several medicinal plants have been extensively studied in order to find more effective and less toxic compounds. We are interested to find out the antioxidant action of aqueous extract of *Ocimum gratissimum (O. gratissimum)* against nicotine toxicity in macrophage, an immunologically important cell. *O. gratissimum* is an important medicinal herb, commonly known as "Ram Tulshi". This plant belongs to the 'Labiaceae' plant family member. The plant is commonly used in folk medicine to treat different diseases, e.g. upper respiratory tract infections, diarrhea, headache, ophthalmic disorders, skin diseases, pneumonia, and also as a treatment for cough, fever, and conjunctivitis. The fresh juice of *O. gratissimum* leaves is used as a mouth antiseptic. It has

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been associated with chemopreventive, anticarcinogenic, free radical scavenging, radio protective and numerous others pharmacological use[5]. Recently we have reported the protective role of methanol extract of *O. gratissimum* against nicotine toxicity[1]. The traditional form of preparation is an aqueous extract of the leaves. Ascorbic acid (AA), a potent antioxidant, is used as beneficial in common medicine practice. AA plays an important role in the defense against oxidative damage, owing to its function as a reducing agent. So, the present study was conducted to evaluate the protective role of aqueous extract of *O. gratissimum* (AE-Og) on free radical generation, antioxidant status, and DNA damage during *in vitro* nicotine toxicity in murine peritoneal macrophages in comparison to the potential antioxidant, ascorbic acid.

2. Materials and methods

2.1. Chemicals and reagents

Hydrogen tartarate salt of nicotine, phorbol myristate acetate (PMA), quercetin, gallic acid, horse heart cytochrome-c, sodium dodecyl sulfate (SDS), 5′, 5′-dithio (bis)-2-nitrobenzoic acid (DTNB), diphenylamine (DPA), standard reduced glutathione, NADPH-Na_4, oxidized glutathione (GSSG) were obtained from Sigma, USA. RPMI 1640, fetal bovine serum (FBS), heparin, ethylene diamine tetra acetate (EDTA) were purchased from Himedia, India. All other chemicals were from Merck Ltd., SRL Pvt. Ltd. Mumbai, India and were of the highest grade available.

2.2. Animals

Experiments were performed using Swiss male mice 6-8 weeks old, weighing 20-25 g. The animals were fed standard pellet diet with vitamins, antibiotic and water were given *ad libitum* and housed in polypropylene cages (Tarson) in the departmental animal house with 12 h light:dark cycle, at the temperature of (25±2) ℃. The animals were allowed to acclimatize for one week. The animals used did not show any sign of malignancy or other pathological processes. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Vidyasagar University.

2.3. Preparation of aqueous extract of O. gratissimum

O. gratissimum was collected from Egra, Puba Medinipur, West Bengal, India in September 2007, in morning. Voucher specimens were deposited at the herbarium of the Dept. of Botany, Vidyasagar University. The fresh aerial part of *O. gratissimum* was dried, blended and extracted with double distilled water (10:1). The mixture was filtered with Whatman filter paper (No. 1) and concentrated at 38 ℃ by a rotary evaporator, then allowed to stand at room temperature over night. The filtration and concentration processes were repeated to yield an aqueous solution. This solution was then centrifuged at 400×g for 10 min and supernatant was freeze dried to obtain the crude aqueous extract (AE-Og).

2.4. Total phenols determination in aqueous extract of O. gratissimum

Total phenols were determined by Folin Ciocalteau reagent [6]. A dilute AE-Og (0.5 mL of 1:10 g/mL) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 mL, 1.0M). The mixtures were allowed to stand for 15 min and the total phenols were determined by Hitachi U2001 spectrophotometer at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/lit solutions of gallic acid in methanol:water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

2.5. Total flavonoids determination in aqueous extract of O. gratissimum

The aluminum chloride colorimetric method was used for flavonoids determination in *O. gratissimum*[7]. Plant aqueous extracts (0.5 mL of 1:10 g/mL) were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1.0M potassium acetate and 2.8 mL of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam Hitachi U2001 UV/Visible spectrophotometer (USA). A calibration curve was prepared by preparing quercetin solutions at concentrations of 10 to 100 μg/mL. Total flavonoid values are expressed in terms of quercetin equivalent (mg/g of dry mass), which is a common reference compound.

2.6. Preparation of drug

Hydrogen tartarate salt of nicotine (Sigma, USA) was dissolved in normal saline (0.9%NaCl) to get the required concentration. The pH of the nicotine solution was adjusted to 7.4 by NaOH[4]. The stock solution of AE-Og was diluted in normal saline to get the required concentrations.

2.7. Isolation of the peritoneal macrophages and cell culture

All efforts were made to minimize animal suffering and to reduce the number of animals used. Macrophages were isolated by peritoneal lavage from male Swiss mice, after 24 h injection of 2 mL of 4% starch according to our previous report[4]. Washing the peritoneal cavity with ice cold phosphate buffer saline (PBS) supplemented with 20 U/mL heparin and 1 mM EDTA performed lavage. Care was taken not to cause internal bleeding while collecting macrophages in the exudates. The cells were then cultured in 60 mm petridishes in RPMI-1640 media supplemented with 10% FBS, 50 μg/mL gentamicin, 50 μg/mL penicillin and 50 μg/mL streptomycin for 24 h at 37 ℃ in a humidified atmosphere of 5% CO_2 – 95% air in CO_2 incubator. Non-adherent cells were removed by vigorously washing three times with ice-cold PBS. Differential counts of the adherent cells used for the experiments were determined microscopically after staining with Giemsa and the cell viability evaluated by Trypan blue exclusion was never below 95%.

2.8. Experimental design and sample preparation

The peritoneal macrophages were divided into 8 groups. Each group contained 6 petridishes (4×10^6 cells in each). The cells of each petridishes of control and experimental groups were maintained in RPMI 1640 media supplemented

with 10% FBS, 50 μg/mL gentamicin, 50 μg/mL penicillin and 50 μg/mL streptomycin at 37 ℃ in a 95% air/5% CO_2 atmosphere in CO_2 incubator.

The following groups were considered for the experiment and cultured for 12 h:

Group I: Control i.e. culture media; Group II: 10 mM nicotine in culture media; Group III: 10 μg AE-Og/mL culture media; Group IV: 10 mM nicotine+1 μg AE-Og/mL culture media; Group V: 10 mM nicotine+5 μg AE-Og/mL culture media; Group VI: 10 mM nicotine+10 μg AE-Og/mL culture media; Group VII: 10 mM nicotine+25 μg AE-Og/mL culture media; Group VIII: 10 mM nicotine+0.01 mM ascorbic acid in culture media. The concentration of nicotine was selected according to our previous lab report[4], and the concentration of ascorbic acid (AA) was selected according to Victor *et al*[7]. After the treatment schedule cells were collected from the petridishes separately and centrifuged at 400×g for 10 min at 4 ℃. Then the supernatant was collected in separate micro centrifuge tubes and the cells were washed twice with 50 mM PBS, pH 7.4. The pellets were lysed with hypotonic lysis buffer (10 mM TRIS, 1 mM EDTA and Titron X-100, pH 8.0) for 45 min at 37 ℃ and then processed for the biochemical estimation. Intact cells were used for superoxide anion generation and NADPH oxidase activity.

2.9. Biochemical estimation

2.9.1. Assessment of superoxide anion ($O_2^{\cdot-}$) generation

Superoxide anion generation was determined by a standard assay[8]. Briefly, 0.1 μg/mL of PMA (Sigma), a potent macrophage stimulant, and 0.12 mM horse heart cytochrome-c (Sigma) were added to isolated cell suspensions after nicotine exposure and washing with PBS. Cytochrome-c reduction by generated superoxide was then determined by spectrophotometric absorbance at a 550 nm wavelength. Results were expressed as nmol of cytochrome-c reduced/min, use the extinction coefficient 2.1×10^4 M^{-1} cm^{-1}.

2.9.2. NADPH oxidase activity

After the treatment schedule the macrophages of different groups were pre-warmed in Krebs Ringer buffer (KRB) with 10 mM glucose at 37 ℃ for 3 min. PMA (0.1 μg/mL) prewarmed at 37 ℃ for 5 min was added, and the reaction was stopped by putting in ice. Centrifugation was carried out at 400 g for 5 min and the resultant pellet was resuspended in 0.34M sucrose. The cells were then lysed with hypotonic lysis buffer. Centrifugation was carried out at 800 ×g for 10 min and the supernatant used to determine enzyme activity. NADPH oxidase activity was determined spectrophotometrically by measuring cytochrome c reduction at 550 nm. The reaction mixture contained 10mM phosphate buffer (pH 7.2), 100 mM NaCl, 1 mM $MgCl_2$, 80 μM cytochrome C, 2 mM NaN_3 and 100 μL of supernatant (final volume 1.0 mL). A suitable amount of NADPH (10-20 μL) was added last to initiate the reaction[9].

2.9.3. Myeloperoxidase (MPO) activity

200 μL of cell lysate was reacted with 200 μL substrate (containing H_2O_2 and OPD) in the dark for 30 min. The blank was prepared with citrate phosphate buffer (pH 5.2) and substrate, in absence of cell free supernatant. The reaction was stopped with addition of 100 μL 2(N) sulfuric acid and readings were taken at 492 nm in a spectrophotometer[10].

2.9.4. Nitrite production (NO)

After the treatment schedule, the cells were then centrifuged at 9 300×g for 20 min. The cell free supernatants were transferred to separate micro centrifuge tube for nitric oxide release assay. To 100 μL of each cell free supernatant, 100 μL of Griess reagent (Containing 1% sulfanilamide in 5% phosphoric acid and 0.1% N-C-1 napthyl ethylene diamine dihydrochloride in 1:1 ratio) were added and incubated at room temperature for 10 min. Reading was taken in a UV spectrophotometer at 550 nm and compared to a sodium nitrite standard curve (values ranging between 0.5 and 25 μM) [11].

2.9.5. Determination of lipid peroxidation (MDA)

Lipid peroxidation was estimated by the method of Ohkawa *et al*[12] in cell lysate. Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), tert-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM $FeSO_4$. After incubating the samples at 37 ℃ for 90 min, the reaction was stopped by adding 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by 1.5 mL of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 mL of 0.8% TBA and further heating the mixture at 95 ℃ for 45 min. After cooling, samples were centrifuged, and the TBA reactive substances (TBARS) were measured in the supernatants at 532 nm by using 1.53×10^5 M^{-1} cm^{-1} as the extinction coefficient. The levels of lipid peroxidation were expressed in terms of nmol/mg protein.

2.9.6. Protein carbonyl contents (PC)

Protein oxidation was monitored by measuring protein carbonyl content by derivatization with 2, 4-dinitrophenyl hydrazine (DNPH)[13]. In general, cell lysate proteins in 50 mM potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2N HCl). Blank samples were mixed with 2N HCl incubated at 1 h in the dark; protein was precipitated with 20% trichloro acetic acid (TCA). Underivatized proteins were washed with an ethanol:ethyl acetate mixture (1:1). Final pellets of protein were dissolved in 6N guanidine hydrochloride and absorbance was measured at 370 nm. Protein carbonyl content was expressed in terms of μmol/mg protein.

2.9.7. Activity of super oxide dismutase (SOD)

SOD activity was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Mestro Del and McDonald[14]. The reaction mixture consisted of 50 mM Tris (hydroxymethyl) aminomethane (pH 8.2), 1 mM diethylenetriamine pentaacetic acid, and 20-50 μL of cell lysate. The reaction was initiated by addition of 0.2 mM pyrogallol, and the absorbance measured kinetically at 420 nm at 25 ℃ for 3 min. SOD activity was expressed as unit/mg protein.

2.9.8. Activity of catalase (CAT)

Catalase activity was measured in the cell lysate by the method of Luck[15]. The final reaction volume of 3 mL contained 0.05M Tris-buffer, 5mM EDTA (pH 7.0), and 10 mM H_2O_2 (in 0.1M potassium phosphate buffer, pH 7.0). About 50 μL aliquot of the lysates were added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. Catalase activity was calculated by using the molar extinction coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2. The level of catalase was expressed in terms of m mol H_2O_2 consumed/min/mg protein.

2.9.9. Determination of reduced glutathione (GSH)

Reduced glutathione estimation in the cell lysate was performed by the method of Moron *et al*[17]. The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2 000 ×g for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 mL of 0.6 mM DTNB was added. After 10 minutes the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as μg of GSH/mg protein.

2.9.10. Oxidized glutathione level (GSSG)

The oxidized glutathione level was measured after derevatization of GSH with 2-vinylpyridine according to the method of Griffith[17]. In brief, with 0.5 mL cell lysate, 2 μL 2-vinylpyidine was added and incubated for 1hr at 37 ℃. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at 1 000×g for 10min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in a spectrophotometer and calculated from a standard GSSG curve.

2.9.11. Activity of glutathione peroxidase (GPx)

The GPx activity was measured by the method of Paglia and Valentine[18]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1U glutathione reductase, and 1 mM reduced glutathione. The sample, after its addition, was allowed to equilibrate for 5 min at 25 ℃. The reaction was initiated by adding 0.1 mL of 2.5 mM H_2O_2. Absorbance at 340 nm was recorded for 5 min. Values were expressed as nmol of NADPH oxidized to NADP by using the extinction coefficient of 6.2×10^3 M^{-1} cm^{-1} at 340 nm. The activity of GPx was expressed in terms of nmol NADPH consumed/min/mg protein.

2.9.12. Activity of glutathione reductase (GR)

The GR activity was measured by the method of Miwa[19]. The tubes for enzyme assay were incubated at 37 ℃ and contained 2.0 mL of 9 mM GSSG, 0.02 mL of 12 mM NADPH-Na_4, 2.68 mL of 1/15M phosphate buffer (pH 6.6) and 0.1 mL of cell lysate. The activity of this enzyme was determined by monitoring the decrease in absorbance at 340 nm. The activity of GR was expressed in terms of nmol NADPH consumed/min/mg protein.

2.9.13. Activity of glutathione-s-transferase (GST)

The activity of GST activity was measured by the method of Habig *et al*[20]. The tubes of enzyme assay were incubated at 25 ℃ and contained 2.85 mL of 0.1M potassium phosphate (pH 6.5) containing 1 mM of GSH, 0.05 mL of 60 mM 1-chloro-2, 4-dinitrobenzene and 0.1 mL cell lysate. The activity of this enzyme was determined by monitoring the increase in absorbance at 340 nm.

2.9.14. DPA assay for DNA fragmentation

The DPA reaction was performed by the method of Paradones *et al*[21]. Perchloric acid (0.5M) was added to the cell pellets containing uncut DNA (resuspended in 200 μL of hypotonic lysis buffer) and to the other half of the supernatant containing DNA fragments. Then two volumes of a solution consisting of 0.088 M DPA, 98% (v/v) glacial acetic acid, 1.5% (v/v) sulphuric acid, and a 0.5% (v/v) concentration of 1.6% acetaldehyde solution were added. The samples were stored at 4 ℃ for 48 h. The reaction was quantified spectrophotometrically at 575 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA.

2.9.15. Protein estimation

Protein was determined according to Lowry *et al*[22] using bovine serum albumin as standard.

2.10. Statistical analysis

The data were expressed as mean±SEM, n=6. Comparisons of the means of control, nicotine, nicotine with different concentration of AE-Og and nicotine with AA group were made by two-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with multiple comparison t-tests, P<0.05 as a limit of significance.

3. Results

3.1. Total phlenolic and flavonoid content in aqueous extract of O. gratissimum

Aqueous extracts of *O. gratissimum* contain high concentrations of phenolic and flavonoid compounds as measured by spectrophotometric methods. AE-Og contained 82.3 mg phenolic compounds /g of *O. gratissimum* powder and 72.5 mg flavonoids/g of *O. gratissimum* powder.

3.2. Superoxide radical generation and NADPH oxidase activity in macrophages

Superoxide anion ($O_2.^-$) generation and NADPH oxidase activity in peritoneal macrophages was significantly (P<0.05) increased in the nicotine treated group by 252.94% and 139.96% respectively, as compared to their control group. AE-Og, present in cell culture media with nicotine, dose responsively decreased the excess $O_2.^-$ generation significantly (P<0.05), when compared to nicotine treated group. Anything over 1 μg/mL AE-Og supplementation significantly (P<0.05) decreased the NADPH oxidase activity, as compared with the nicotine treated group, where as 10 μg/mL AE-Og exerted the maximum protective effect. Only 10 μg/mL AE-Og treatment slightly increased both the $O_2.^-$ generation and NADPH oxidase activity, as compared to their respective control groups. ascorbic acid (AA) had more power to decrease the excess $O_2.^-$ generation and NADPH oxidase activity than AE-Og (Figure 1).

3.3. Nitrite production and myeloperoxidase activity in macrophages

Nitrite (NO) production and MPO activity in peritoneal macrophages in different group are shown in Figure 2. NO production and MPO activity in macrophages both were significantly (P<0.05) increased in nicotine treated group by 93.98% & 79.76% respectively, as compared to their respective control group. AE-Og (5 μg/mL, 10 μg/mL & 25 μg/mL) and AA supplementation both decreased the excess NO production and MPO activity significantly (P<0.05)

compared to nicotine treated group. Out of the four different concentrations of AE-Og, 10 μg/mL AE-Og showed the highest protective effect to decrease the NO production and MPO activity in nicotine treated murine macrophages, slightly less than the AA effect.

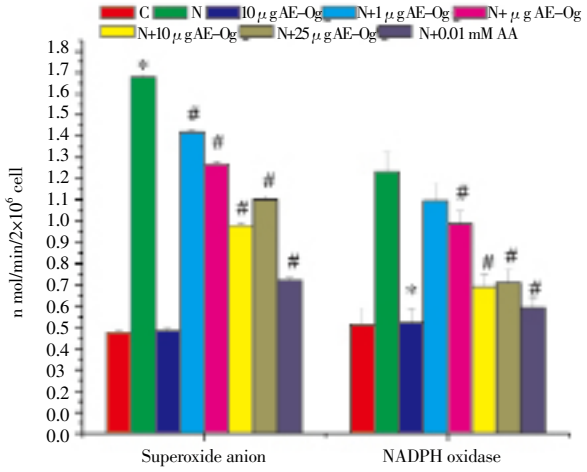

Figure 1. Superoxide anion generation and NADPH oxidase activity in control (C), nicotine (N), AE-Og, nicotine+different concentrations of AE-Og and nicotine+AA treated peritoneal macrophages.
Values are expressed as mean±SEM, n=6; * Significant difference (P<0.05) compared to control group, # Significant difference (P<0.05) compared to nicotine treated group.

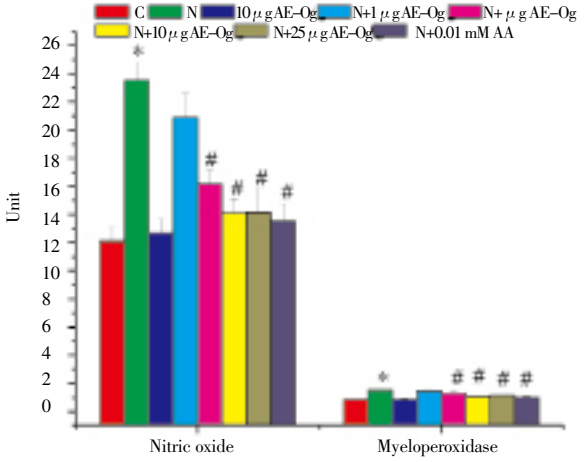

Figure 2. Nitric oxide generation and myeloperoxidase activity in control (C), nicotine (N), AE-Og, nicotine+different concentrations of AE-Og and nicotine+AA treated peritoneal macrophages.
Values are expressed as mean±SEM, n=6; * Significant difference (P<0.05) compared to control group, # Significant difference (P<0.05) compared to nicotine treated group; Unit: Nitrite generation (μmol/mg protein); myeloperoxidase (unit/mg protein).

3.4. Lipid peroxidation and protein oxidation level in macrophages

Lipid peroxidation and protein oxidation in peritoneal macrophages was measured in terms of MDA and PC levels in cells. MDA level and PC content was significantly (P<0.05) increased in nicotine treated murine macrophages by 221.60% and 146.98%, respectively, as compared to their control group. Only AE-Og treatment slightly increased MDA level but decreased PC content as compared to their respective control group, but there was no significant difference. Supplementation with AE-Og decreased the MDA level and PC content significantly (P<0.05) in a concentration dependent manner, except 1 μg/mL AE-Og in the case of MDA level. 9.40%, 29.94%, 52.20%, 50.86%, & 54.16% MDA level and 17.21%, 23.29%, 36.88%, 40.97%, & 46.55% PC content were decreased with supplementation of 1 μg/mL, 5 μg/mL, 10 μg/mL, 25 μg/mL AE-Og and 0.01 mM ascorbic acid (AA) respectively, as compared with nicotine treated group. In both cases 10 μg/mL AE-Og showed the highest protective effect in murine macrophages and was more or less equal to the effect of AA supplementation (Figure 3).

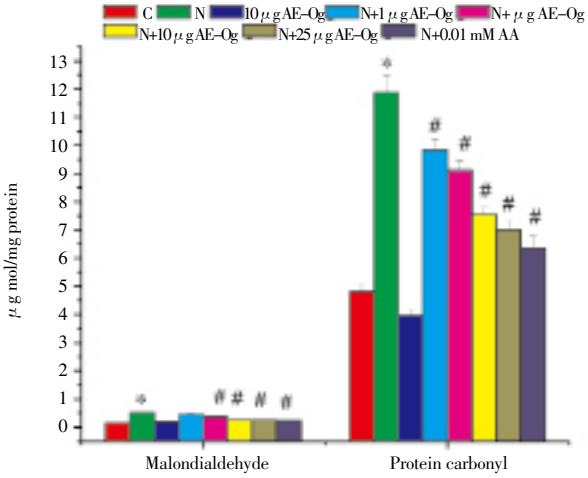

Figure 3. Malondialdehyde and protein carbonyl level in control (C), nicotine (N), AE-Og, nicotine + different concentrations of AE-Og and nicotine+AA treated peritoneal macrophages.
Values are expressed as mean±SEM, n=6; * Significant difference (P<0.05) compared to control group, # Significant difference (P<0.05) compared to nicotine treated group.

3.5. Antioxidant status in macrophages

The GSH, GSSG level and SOD, catalase, GPx, GR, GST activity were measured to understand the antioxidant status of different group of macrophages. The level of GSH and other enzymatic antioxidant (SOD, CAT, GPx, GR & GST) activity was decreased significantly (P<0.05) in the nicotine treated group, when compared to their respective control group. These antioxidant activities were significantly (P<0.05) concentration dependently increased in the AE-Og supplemented group (except GPx and GST activity with 1 μg/mL AE-Og), as compared to their nicotine treated group. AA significantly (P<0.05) increased these antioxidant status indicators in macrophages, as compared to their nicotine treated group. Among the different concentrations of AE-Og, 10 μg/mL was the most effective dose against the nicotine toxicity. Only AE-Og treatment enhanced the SOD, GPx, GR, GST activity and GSH level but slightly decreased the catalase activity. The GSSG level was increased significantly (P<0.05) in nicotine treated macrophage, as compared with the control group. Supplementation with AE-Og (10 μg/mL & 25 μg/mL) and AA significantly (P<0.05) decreased the GSSG level, when compared with the nicotine

treated group (Figure 4-6).

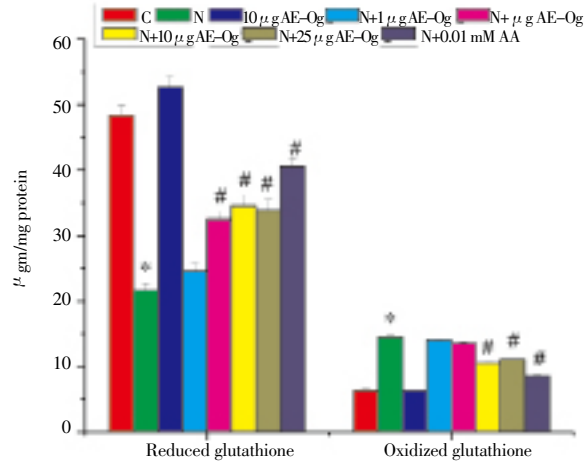

Figure 4. Reduced glutathione and oxidized glutathione level in control (C), nicotine (N), AE-Og, nicotine + different concentrations of AE-Og and nicotine+AA treated peritoneal macrophages.
Values are expressed as mean±SEM, n=6; * Significant difference (P<0.05) compared to control group, # Significant difference (P<0.05) compared to nicotine treated group.

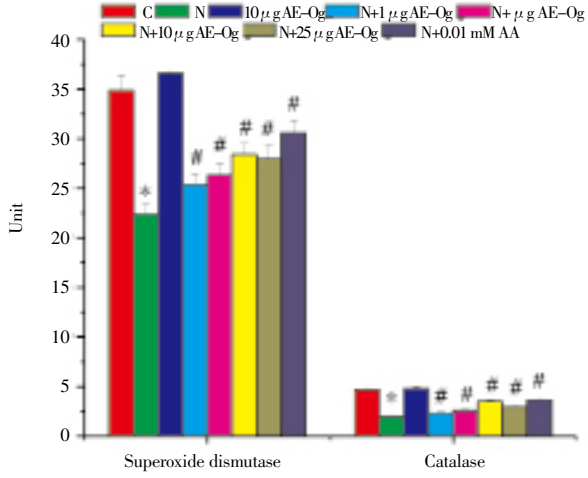

Figure 5. Superoxide dismutase and catalase activity in control (C), nicotine (N), AE-Og, nicotine + different concentrations of AE-Og and nicotine + AA treated peritoneal macrophage.
Values are expressed as mean±SEM, n=6; * Significant difference (P<0.05) compared to control group, # Significant difference (P<0.05) compared to nicotine treated group, Unit: superoxide dismutase (unit/mg protein); catalase (mmol H_2O_2 decomposed/min/mg protein).

3.6. DNA fragmentation in macrophages

DNA fragmentation in all group of macrophage was evaluated by DPA assay spectrophotometrically. *In vitro* nicotine produced the 88.48% fragmented DNA, whereas the control group showed negligible 5.39% fragmented DNA in peritoneal macrophage. AE-Og treatment alone produced 5.16% fragmented DNA, also negligible. Supplementation of 1 μg/mL, 5 μg/mL, 10 μg/mL, & 25 μg/mL AE-Og decreased the DNA fragmentation in a dose dependent manner and produced 53.51%, 46.52%, 22.38%, 25.16% DNA fragmentation respectively in macrophage. AA supplementation also decreased the DNA fragmentation compared to the nicotine treated group and produced 18.22% fragmented DNA. Among the different concentrations of AE-Og, 10 μg/ml was the most effective but AA was more protective role than AE-Og (Figure 7).

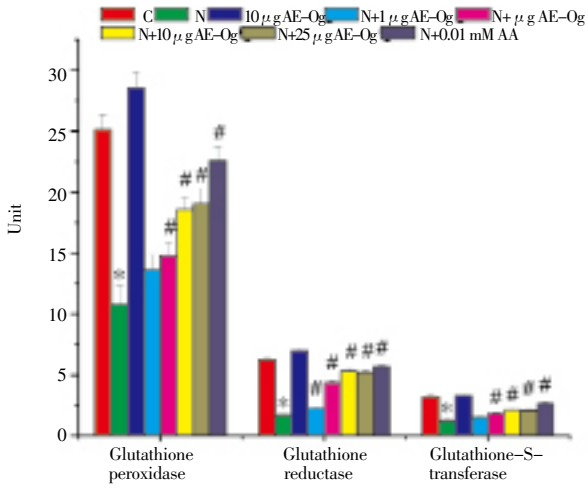

Figure 6. Glutathione peroxidase, glutathione reductase, and glutathione-S-transferase activity in control (C), nicotine (N), AE-Og, nicotine + different concentrations of AE-Og and nicotine + AA treated peritoneal macrophage.
Values are expressed as mean±SEM, n=6, * Significant difference (P<0.05) compared to control group, # Significant difference (P<0.05) compared to nicotine treated group; Unit: glutathione peroxidase & glutathione reductase (nmol NADPH consumed/min/mg protein); glutathione-S-transferase (mmol/min/mg protein).

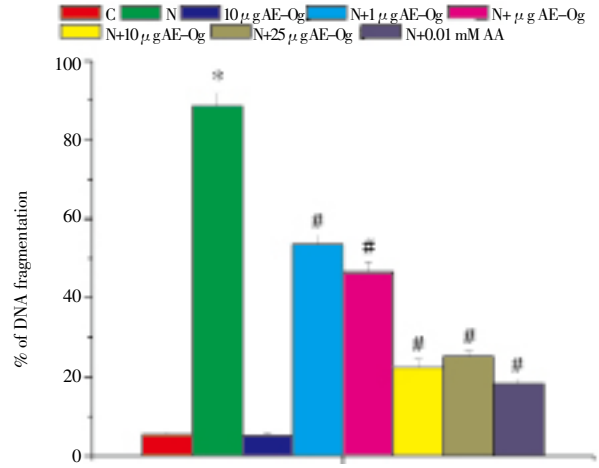

Figure 7. DNA fragmentation in control (C), nicotine (N), AE-Og, nicotine + different concentrations of AE-Og and nicotine+AA treated peritoneal macrophage.
Values are expressed as mean±SEM, n=6; * Significant difference (P<0.05) compared to control group, # Significant difference (P<0.05) compared to nicotine treated group.

4. Discussion

The results of the present study indicate that *in vitro* nicotine-induced cellular damage in mice peritoneal macrophages, as evidenced by enhanced superoxide anion generation, NADPH oxidase activity, MPO activity, MDA and PC level and decreased GSH level and also decreased SOD, CAT, GPx, GR and GST activity, are ameliorated by administration of AE-Og and AA. Moreover DNA damage assessed by DPA assay due to nicotine treatment was also prevented by AE-Og and AA supplementation.

Oxidative stress is the result of an imbalance between the generation of ROS and the antioxidant system in favor of the former. Macrophage use ROS to carry out many functions. They need appropriate levels of intracellular antioxidants to eliminate the harmful effect of oxidative stress[23]. Nicotine, a major toxic component of cigarette smoke, is a well established procarcinogen. In our present investigation, significantly (P<0.05) increased superoxide anion generation and activation of NADPH oxidase were observed in nicotine exposed peritoneal macrophages. The activated NADPH oxidase transports electrons from NADPH on the cytoplasmic side of the membrane to oxygen in the extracellular fluid to form $O_2\cdot^-$[24]. This $O_2\cdot^-$ leads to oxidative damage of macromolecules including lipid, protein, DNA and antioxidants present in the cell. In this study, the AE-Og caused concentration dependent decreased activity of NADPH oxidase and superoxide radical generation in peritoneal macrophages. Thus *O. gratissimum* is protecting the oxidative immune cell damage by reducing the activity of NADPH oxidase and as a result decrease the $O_2\cdot^-$ generation.

It is well known that ROS cause damage to membrane lipids, a process of lipid peroxidation. After lipid peroxidation, biological consequences such as disturbance of membrane organization, and secondary lipid peroxidation products occur. Many of these products 4-hydroxynonenals (HNE) or other aldehydes, such as MDA, exert similar toxic effects, which can prolong and potentiate the primary free radical initiated damage[25]. Macrophages are ubiquitous mononuclear phagocytes in mammalian tissues. The peritoneal macrophages are representative of other macrophage populations. The peritoneal macrophages are highly susceptible to oxidative damage due to the presence of high percent polyunsaturated fatty acids in their plasma membrane and high production of ROS, which is part of their normal function. The present study showed elevated levels of lipid peroxidation products up to 221.6% above basal values in peritoneal macrophages after *in vitro* nicotine treatment. Free radical generation through nicotine toxicity can also react with protein in addition to lipid. In our study, nicotine induced oxidative modified proteins (PC) were increased significantly. Thus, free radical ablation with the antioxidant agents seem to be beneficial for preventing the damage of lipid and protein. In our previous studies, we have shown that aqueous extract of *Andrographis paniculata* Nees, as antioxidant agent, protected experimental rat tissues and lymphocytes against nicotine-induced oxidative damage[3,26]. Similarly, in the present study, aqueous extract of *O. gratissimum* caused concentration dependent significant (P<0.05) protection of MDA production and PC content indicating a reduction in lipid peroxidation, protein oxidation and cellular injury in macrophage, thus playing a protective role against oxidative immune cell damage preserving the cellular integrity.

Nitric oxide plays an important role in a diverse range of physiological processes. NO reacts with the superoxide anion to generate peroxynitrite, which is a selective oxidant, and nitrating agent that interacts with numerous biological molecules, thereby damaging them[27]. In this present investigation increased level of NO in cell culture supernatant of nicotine administered peritoneal macrophage were observed. AE-Og and AA both modulated the nicotine induced NO production in peritoneal macrophage significantly. Various studies have shown that nitric oxide synthesis is high in tumor tissue and in plasma, which can be related to an alteration in oxidant-antioxidant potential[28]. Thus, higher level of NO in the nicotine treated peritoneal macrophage may be due to high production of free radicals. Co-administration of AE-Og and AA with nicotine reveals the role of antioxidants to prevent the formation of NO in the immune cell.

ROS also can generate hypochlorous acid (HOCl) in the presence of macrophage derived MPO and initiate the deactivation of antiproteases and the activation of latent proteases, that leads to the tissue damage. In our study, AE-Og inhibited the MPO activity that was increased by nicotine treatment; suggesting a protective effect of *O. gratissimum*. AA also significantly (P<0.05) reduced increased MPO activity attributable to nicotine administration. These results suggest that the cellular antioxidants reached concentrations in macrophage sufficient to exert antioxidant effects. Thus, in addition to the cellular antioxidant system, AE-Og and AA may indirectly protect macrophage from nicotine toxicity.

DNA damage was significantly (P<0.05) reduced in peritoneal macrophage in the AE-Og and AA treated groups. The phenolics present in AE-Og may bind to DNA, and its molecules could be positioned in such a way as to effectively scavenge reactive intermediates that approach the critical sites on DNA, or phenolics may directly interact with the ultimate reactive metabolites of carcinogen by donating their electrons, and rendering it inactive.

Our results also showed decreased activities of enzymatic antioxidants SOD, CAT, GPx, GR and GST and the levels of non-enzymatic antioxidant GSH in nicotine-treated murine peritoneal macrophage. ROS generated in tissues are normally scavenged by enzymatic and non-enzymatic antioxidants[29]. The antioxidant defense system protects the aerobic organism from the deleterious effects of reactive oxygen metabolites. Glutathione is a crucial component of the antioxidant defense mechanism and it functions as a direct reactive free radical scavenger[30]. In our study, the decreased GSH level may be due to increasing level of lipid oxidation products which may be associated with less availability of NADPH required for the activity of GR to transform GSSG to GSH[31], due to the increasing production of ROS at a rate that exceeds the ability to regenerate GSH in macrophages with *in vitro* nicotine treatment. The decreased level of GSH and increased level of GSSG in nicotine-treated macrophages of the present study may be due to enhanced utilization during detoxification of nicotine. GPx and CAT, which act as preventive antioxidants and SOD, a chain breaking antioxidant, play an important role in protection against the deleterious effects of lipid peroxidation[32]. Depletion in the activities of SOD, CAT, GPx, and GR in nicotine-treated macrophages may be due to decreased synthesis of enzymes or oxidative inactivation of enzyme protein. GST mainly detoxifies electrophilic compounds[33], and has a well-established role in protecting cells from mutagens and carcinogens as a free radical scavenger along with glutathione. In the present study, the depletion of GSH level and decreased activity of GSH-dependent enzymes, i.e. GPX, GR, and GST in peritoneal macrophages on *in vitro* treatment with nicotine administration may be due to increased utilization to scavenge the free radical generation. In the present study, increased lipid peroxidation associated with decreased antioxidant status in nicotine-treated macrophages can therefore be related to insufficient antioxidant potential. Our results also demonstrated that, *O. gratissimum* extract and AA protects the cell through enhancing the both enzymatic and non enzymatic cellular antioxidants like SOD, CAT, GPx, GR, GST and GSH, more or less near to control level. One of the possible reasons behind it may be the antioxidative property of the AE-Og. Our results also revealed that AE-Og contains high level of phenolic and flavonoid compound and

protects the macrophage during *in vitro* nicotine induced lipid peroxidation, PC, free radical generation (Superoxide anion generation, NADPH oxidase activity, MPO activity, NO production) and DNA damage. Beside that, AE-Og also increased the antioxidant enzymes status possibly due to flavonoids present in it, which may exert a stimulatory action on transcription and gene expression of certain antioxidant enzymes[34]. Phenolic compounds act as free radical scavengers by virtue of their hydrogen donating ability[35]. Therefore, presence of phenolic compound in AE-Og may boost the antioxidant property of *O. gratissimum*.

In conclusion, we demonstrated that *O. gratissimum* and ascorbic protected the murine peritoneal macrophages from nicotine toxicity by decreasing free radical generation, lipid, protein and DNA damage and also increased antioxidant status. Hence, AE-Og may be useful as a potent free radical scavenger and antioxidative product and may be a potential therapeutic agent against nicotine toxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

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