Research Paper

Methanol extract of *Ocimum gratissimum* protects murine peritoneal macrophages from nicotine toxicity by decreasing free radical generation, lipid and protein damage and enhances antioxidant protection

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**Abbreviations:** AA, ascorbic acid; BHP, tetra butyl hydro peroxide; CAT, catalase; COX-2, cyclooxygenase-2; DNA, deoxyribonucleic acid; DNPH, 2, 4-dinitrophenylhydrazine; DTNB, 5', 5'-dithio (bis)-2-nitrobenzoic acid; EDTA, ethylene diamine tetra acetate; FBS, fetal bovine serum; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-s-transferase; KRB, krebs ringer buffer; MDA, malondialdehyde; ME-Og, methanol extract of *Ocimum gratissimum* linn; MPO, myeloperoxidase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PBS, phosphate buffer saline; PC, protein carbonyls; PMA, phorbol mirested aceted; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; SSA, sulfosalicylic acid; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; TCA, trichloro acetic acid

**Key words:** *Ocimum gratissimum*, nicotine, free radical, antioxidants, murine peritoneal macrophage

In the present study, methanol extract of *Ocimum gratissimum* Linn (ME-Og) was tested against nicotine-induced murine peritoneal macrophage in vitro. Phytochemical analysis of ME-Og shown high amount of flavonoid and phenolic compound present in it. The cytotoxic effect of ME-Og was studied in murine peritoneal macrophages at different concentrations (0.1 to 100 μg/ml) using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) method. To establish the protective role of ME-Og against nicotine toxicity, peritoneal macrophages from mice were treated with nicotine (10 mM), nicotine + ME-Og (1 to 25 μg/ml) for 12 h in culture media. The significantly (p < 0.05) increased super oxide anion generating, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, myeloperoxidase (MPO) activity, lipid peroxidation, protein carbonyls, oxidized glutathione levels were observed in nicotine-treated group as compared to control group; those were significantly (p < 0.05) reduced in ME-Og supplemented groups in concentration dependent manner. More over, significantly (p < 0.05) reduced antioxidant status due to nicotine exposure was effectively ameliorated by ME-Og supplementation in murine peritoneal macrophages. Among the different concentration of ME-Og, maximum protective effect was observed by 25 μg/ml, which does not produce significant cell cytotoxicity in murine peritoneal macrophages. These findings suggest the potential use and beneficial role of *O. gratissimum* as a modulator of nicotine-induced free radical generation, lipid-protein damage and antioxidant status in important immune cell, peritoneal macrophages.

**Introduction**

Several medicinal plants employed in folk medicine, mainly in communities with inadequate conditions of public health, have been extensively studied in order to find less toxic compound more effectiveness. We are interested to find out the antioxidant property of *Ocimum gratissimum* Linn which is commonly used in folk medicine. *O. gratissimum* is an important medicinal herb which is commonly known as “Ram Tulshi.” It belongs to the family of ‘Labiaceae’. *O. gratissimum* is associated with chemo-preventive, anti-carcinogenic, free radical scavenging, radio protective and numerous others pharmacological use.1 *O. gratissimum* is used to treat different diseases, e.g., upper respiratory tract infections, diarrhea, headache, ophthalmic, skin diseases, pneumonia, and also as a treatment for cough, fever and conjunctivitis.2,3 Earlier reports have shown the smooth muscle contracting lipid soluble principles, and antimutagenic activity in organic solvent extracts of *O. gratissimum*
leaves. This medicinal plant has also potential role as antibacterial, antifungal, antimicrobial, anthelmintic, and in vitro antidermatophytic agent. The aqueous leaf extract and seed oil showed anti-proliferative and chemo-preventive activity on HeLa cells. Nicotine is an alkaloid which is composed of a pyridine and a pyrroline ring. It is found in the plant kingdom throughout a wide range of families. Use of tobacco smoking, cigarette smoking, tobacco chewing, various tobacco products and also nicotine replacement therapies are the main important sources of human exposure to nicotine, as it is the main active ingredient. Nicotine has been recognized to result in oxidative stress by inducing the generation of reactive oxygen species (ROS). These ROS in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation, protein oxidation and DNA damage. Lipid peroxidation is known to cause cellular injury by inactivation of membrane enzymes and receptors, polymerization of polysaccharide, as well as protein cross-linking and fragmentation. The immune cells use ROS for carrying out their normal functions but an excess amount of ROS can attack cellular components that lead to cell damage. Previous reports from our laboratory and by other distinguished researcher have shown that nicotine administration results in the imbalance of prooxidant/antioxidant status in different tissues of Wister rats. In vitro experiments by many researchers were also shown that nicotine severely damages the DNA and imbalance the prooxidant/antioxidant status in lymphocytes. In vitro experiment with mice peritoneal macrophages were also established that nicotine dose dependently generate superoxide radical, damage the lipid and protein, and diminish the antioxidant status in murine macrophages. Peroxidized lipids, depletion of glutathione and glutathione dependent enzymes, and other antioxidants are considered to be important biological markers, as they may have a role in the development of oral cancer.

Macrophages are ubiquitous mononuclear phagocytes in mammalian tissues. The peritoneal macrophages are representative of other macrophage populations and also easily available in mice in greater amounts than blood phagocytes (monocytes or neutrophils). The immune cell functions are specially linked to neutrophils. The immune cell functions are specially linked to neutrophils and therefore preserving their adequate function. In our previous lab report, it was clearly established that nicotine can damage the murine peritoneal macrophages. Therefore, the present study was performed to find out a new therapeutic approach against nicotine toxicity in murine peritoneal macrophages.

The present study was conducted to evaluate the protective role of methanol extract of O. gratissimum (ME-Og) on free radical generation, lipid-protein damage and antioxidant status during in vitro nicotine toxicity in murine peritoneal macrophages.

Figure 1. Phenolic and flavonoid content in methanol extract of O. gratissimum (ME-Og) were determined in UV-Vis spectrophotomer using quercetin (in case of flavonoid), and gallic acid (in case of phenolics) as the standard. The results indicate that flavonoid is higher amount in ME-Og than phenolic compound. The experiments were repeated six times, yielding similar results and data are shown as mean ± SEM.

Results

Total phenolic and flavonoid content in ME-Og extract. The Result clearly proposed that ME-Og contains high phenolic and flavonoid compound that was measured by spectrophotometric method. ME-Og contains 61.72 mg phenolic compound/g of O. gratissimum powder, and 251.83 mg flavonoid/g of O. gratissimum powder (Fig. 1).

Determination of cell cytotoxicity by ME-Og. The cytotoxic effect of the ME-Og was studied in murine peritoneal macrophages with increasing concentrations of ME-Og ranging from 0.1 μg/ml to 100.00 μg/ml using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) method. The results indicated that, treatment of peritoneal macrophages with 10, 25, 50 and 100.00 μg/ml for 24 h led to 9.75%, 11.41%, 25.37% and 46.82% reduction in cell survivability, respectively (Fig. 2). But, there is no significant difference in cell survivability among 0.1 μg/ml to 25 μg/ml of ME-Og treatment and so, it is the highest concentrations of ME-Og, which does not produce any significant damage to murine peritoneal macrophages.

Super oxide radical generation and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Superoxide anion (O2•−) generation and NADPH oxidase activity in peritoneal macrophages was significantly (p < 0.05) increased in nicotine treated group by 102.71% and 117.01% respectively, as compared to their control group. Only ME-Og (25.0 μg/ml) treatment decreased the O2•− generation (22.97%) and NADPH oxidase activity (11.20%), as compared to their respective control group. ME-Og supplementation with nicotine could concentration dependently decreased the excess O2•− generation significantly (p < 0.05), when compared to nicotine treated group. Where, 1 μg/ml ME-Og supplementation could decreased...
increased in nicotine treated group by 116.71% as compared to peritoneal macrophage. MPO activity is significantly (p < 0.05) important determinant to establish the free radical generation in cellular system that leads to oxidative damage. So, it is an important antioxidant in cellular system. In this study, we have measured both reduced and oxidized form of glutathione. Reduced glutathione (GSH) level was decreased (66.58%) significantly (p < 0.05) in nicotine treated macrophage, as compared with control group. Supplementation with ME-Og increased the oxidized glutathione (GSSG) level in concentration dependent manner (22.72%, 59.93%, 74.40% and 77.50%), when compared with nicotine treated group (Table 3). Only ME-Og treatment (25 μg/ml) slightly increased GSH level (4.33%) as compared to their respective control group.

The GSSG level was increased (53.77%) significantly (p < 0.05) in nicotine treated macrophage, as compared with control group. Supplementation with ME-Og decreased the GSSG level in concentration dependent manner (3.99%, 4.09%, 13.26% and 17.59%), when compared with nicotine treated group (Table 3). Only ME-Og treatment (25 μg/ml) slightly increased GSSG level (2.65%) as compared to their respective control group. The redox ratio was decreased (78.37%) significantly (p < 0.05) in nicotine treated macrophage, as compared with control group. Supplementation with ME-Og increased the redox ratio in concentration dependent manner (28.18%, 66.94%, 101.55% and 116.75%), when compared with nicotine treated group (Table 3). Only ME-Og treatment (25 μg/ml) slightly increased the redox ratio (1.11%) as compared to their respective control group.

Antioxidant enzymes status. The super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-transferase (GST) activity were measured to understand the antioxidant status of different group of macrophages. The activity of different enzymatic antioxidant (SOD, CAT, GPx, GR & GST) activity was decreased significantly (p < 0.05) when compared to their respective control. These antioxidant activities were significantly (p < 0.05) raised in ME-Og supplemented group in concentration dependent manner (except GPx and GR activity with 25 μg/ml ME-Og), as compared to their nicotine treated group. Among the different concentrations of ME-Og, 25 μg/ml ME-Og shows the highest protective effect to decrease the MPO activity in nicotine treated murine peritoneal macrophages.

Lipid peroxidation and protein oxidation. Lipid peroxidation and protein oxidation are the two important determinants to assess the cellular damage. Lipid peroxidation and protein oxidation in peritoneal macrophages was measured in terms of malondialdehyde (MDA) and protein carbonyls (PC) respectively. MDA level and PC content was significantly (p < 0.05) increased in nicotine treated murine macrophages by 200.56% and 135.54%, respectively, as compared to their control group. Only ME-Og treatment slightly increased MDA level (14.04%) but decreased PC content (1.58%) as compared to their respective control group, but there was no significant difference. Supplementation of ME-Og can decreased the MDA level and PC content significantly (p < 0.05) in concentration dependent manner, except 25 μg/ml ME-Og in case of PC level. 7.10%, 12.89%, 37.38% and 39.81% MDA level and 1.34%, 21.81%, 24.83% and 24.14% PC content were decreased with supplementation of 1 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml ME-Og as compared with nicotine treated group (Table 2).

**Discussion**

It is evident from our study that, in vitro nicotine-induced cellular damage in mice peritoneal macrophages is associated with enhanced superoxide anion generation, NADPH oxidase activity, MPO activity, MDA level, PC level, GSSG level and decreased

![Figure 2. Dose-response curve of the effect of methanol extract of O. gratissimum (ME-Og) on cell cytotoxicity as well as viability of peritoneal macrophage.](image-url)
GSH level and as well as decreased enzymatic antioxidant (SOD, CAT, GPx, GR and GST) activity, which are protected by co-administration of ME-Og (Tables 1–5), that containing flavonoid and phenolic compound (Fig. 1). Beside that, we observed cytotoxic profile of ME-Og in murine peritoneal macrophages by MTT assay (Fig. 2) and determined its least comparative cytotoxic concentration to be 25 μg/ml, whereas concentrations higher than 25 μg/ml showed significant increased cytotoxicity. More over, microscopic examinations of treated murine peritoneal macrophage reveal that, nicotine can damage the peritoneal macrophage which is protected by supplementation of ME-Og (Fig. 3).

Imbalance between the generation of reactive oxygen species (ROS) and the antioxidant system causes oxidative stress. Macrophage, an immune cell, uses ROS to carry out many of its functions. It needs appropriate levels of intracellular antioxidants to eliminate the harmful effect of ROS.28 In our present investigation, significantly (p < 0.05) increased the generation of superoxide anion and activation of NADPH oxidase was observed in peritoneal macrophages due to in vitro exposure of nicotine (Table 1). The activated NADPH oxidase transports electrons from NADPH on the cytoplasmic side of the membrane to oxygen in the extracellular fluid to form O2•−.29 This O2•− leads to oxidative damage of macromolecules including lipid, protein, DNA and antioxidant enzymes. Beside that, decreased NADPH oxidase activity was observed in the ME-Og supplementation group, as a result decreased O2•− was found in this present study. Thus O. gratissimum protect the immune cell through reducing the NADPH oxidase activity and excess O2•− generation. In the presence of macrophage derived MPO, ROS generates hypochlorous acid (HOCI) and initiate the deactivation of antiproteases and the activation of latent proteases, that leads to the tissue damage.30

In our study, ME-Og supplementation also inhibits the MPO activity which was increased due to nicotine toxicity; suggest that protective role of O. gratissimum (Table 1).

Nicotine is highly addictive alkaloid and has been reported to induce oxidative stress both in vitro and in vivo.25,31 Previous studies have suggested that, superoxide anion and hydrogen peroxide are the main source of nicotine induced free radicals depleting the cellular antioxidants.32 Moreover, it is well known that, ROS causes the damage to membrane lipids, a process of lipid peroxidation. After lipid peroxidation, its biological consequences such as disturbance of membrane organization, etc., secondary lipid peroxidation products are formed. 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.33,34 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.35 The present study showed elevated levels of lipid peroxidation products up to 200.56% above basal values in peritoneal macrophages after in vitro nicotine treatment (Table 2). 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 (p < 0.05). Beside that, ME-Og caused concentration dependent significant (p < 0.05) protection of MDA production and PC content, indicating a reduction in lipid peroxidation and an increase in the amount of antioxidants.
Our results also showed decreased activities of enzymatic antioxidants like SOD, CAT, GPx, GR and GST and the levels of non-enzymatic antioxidant, GSH in nicotine-treated murine peritoneal macrophage (Tables 3–5). Glutathione is a crucial component of the antioxidant defense mechanism, and it functions as a direct reactive free radical scavenger. In this 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 glutathione reductase (GR) to transform GSSG to GSH 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 (Table 3). 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. Depletion in the activities of SOD, CAT, GPx and GST in nicotine-treated peritoneal macrophages may be due to decreased synthesis of enzymes or oxidative inactivation of enzyme protein. Glutathione-S-transferase (GST) mainly detoxifies electrophilic compounds 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 GSH level and GSH-dependent enzymes activity decreased (GPx, GR and GST) in peritoneal macrophages on in vitro nicotine administration may be due to increased utilization to scavenge the free radical generation. Therefore, increased lipid peroxidation associated with decreased antioxidant status in nicotine-treated macrophages can be related to insufficient antioxidant potential. Our results also demonstrated that, ME-Og can 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 reason behind it, is may be the antioxidative property of the ME-Og, that contains high level of phenolic and flavonoid compound (Fig. 1) and protect the macrophage during in vitro nicotine induced lipid peroxidation, PC, free radical generation (Superoxide anion generation, lipid peroxidation, MPO activity). Beside that, ME-Og supplementation increased the antioxidant enzymes status due to flavonoid present in it, which may exert a stimulatory action on transcription and gene expression of certain antioxidant enzymes. Phenolic compounds act as free radical scavengers by virtue of their hydrogen donating ability. Therefore, presence of phenolic compound in ME-Og may boost up the antioxidant property of *O. gratissimum*.

### Table 3 Reduced glutathione (GSH), oxidized glutathione (GSSG) and redox ratio (GSH/GSSG) in murine peritoneal macrophages of different experimental groups

| Parameter | Reduced glutathione (μg/gm/mg protein) | Oxidized glutathione (μg/gm/mg protein) | GSH/GSSG ratio |
|-----------|----------------------------------------|---------------------------------------|----------------|
| Control   | 54.53 ± 2.094                          | 10.188 ± 0.589                       | 5.382 ± 0.129  |
| Nicotine  | 18.225 ± 1.04*                         | 15.666 ± 0.422*                      | 1.164 ± 0.066* |
| 25.0 μg/ml ME-Og | 56.892 ± 1.168                      | 10.458 ± 0.329                       | 5.469 ± 0.217  |

| Parameter | Reduced glutathione (μg/gm/mg protein) | Oxidized glutathione (μg/gm/mg protein) | GSH/GSSG ratio |
|-----------|----------------------------------------|---------------------------------------|----------------|
| N + 1.0 μg/ml ME-Og | 22.365 ± 0.616*                      | 15.04 ± 0.292                         | 1.492 ± 0.062* |
| N + 5.0 μg/ml ME-Og | 29.148 ± 0.702*                      | 15.025 ± 0.234                       | 1.943 ± 0.064* |
| N + 10.0 μg/ml ME-Og | 31.785 ± 1.103*                     | 13.588 ± 0.357*                      | 2.346 ± 0.1* |
| N + 25.0 μg/ml ME-Og | 32.35 ± 1.1*                        | 12.91 ± 0.48*                        | 2.523 ± 0.125* |

### Table 4 Superoxide dismutase (SOD) and catalase (CAT) activity in murine peritoneal macrophages of different experimental groups

| Parameter | Superoxide dismutase (units/mg protein) | Catalase (mmol H₂O₂ decompose/min/mg protein) |
|-----------|----------------------------------------|---------------------------------------------|
| Control   | 34.102 ± 0.295                         | 4.802 ± 0.245                               |
| Nicotine  | 20.263 ± 0.426*                        | 1.812 ± 0.153*                             |
| 25.0 μg/ml ME-Og | 27.95 ± 0.773*             | 2.726 ± 0.209*                             |

After the treatment schedule, the GSH and GSSG levels were measured spectrophotometrically followed by GSH/GSSG ratio was calculated. The results indicate that, GSH level and redox ratio are significantly decreased, but GSSG level is significantly increased in nicotine treated cells than control; which are significantly modulated by supplementation of methanol extract of *O. gratissimum* (ME-Og). There is no significant alteration of GSH level, GSSG level, and GSH/GSSG ratio, when cells are treated with only ME-Og. So, ME-Og is protecting murine peritoneal macrophages against nicotine toxicity through modulating the glutathione level. Values are expressed as mean ± SEM, n = 6. *indicates significant difference (p < 0.05) compared to control group. #indicates significant difference (p < 0.05) compared to nicotine treated group.

After the treatment schedule, SOD and CAT activity were measured spectrophotometrically. The results indicate that, SOD and CAT are significantly decreased in nicotine treated group than control group; which are significantly enhanced by supplementation of methanol extract of *O. gratissimum* (ME-Og). More over, SOD and CAT activity are enhanced in only ME-Og treated cells than cells in control group. So, ME-Og can boost up these two antioxidant enzyme and protect cell from nicotine toxicity. Values are expressed as mean ± SEM, n = 6. *indicates significant difference (p < 0.05) compared to control group. #indicates significant difference (p < 0.05) compared to nicotine treated group.

### Table 5 Reduced glutathione (GSH), oxidized glutathione (GSSG) and redox ratio (GSH/GSSG) in murine peritoneal macrophages of different experimental groups

| Parameter | Reduced glutathione (μg/gm/mg protein) | Oxidized glutathione (μg/gm/mg protein) | GSH/GSSG ratio |
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| Nicotine  | 18.225 ± 1.04*                         | 15.666 ± 0.422*                      | 1.164 ± 0.066* |
| 25.0 μg/ml ME-Og | 56.892 ± 1.168                      | 10.458 ± 0.329                       | 5.469 ± 0.217  |

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| N + 25.0 μg/ml ME-Og | 32.35 ± 1.1*                        | 12.91 ± 0.48*                        | 2.523 ± 0.125* |

### Table 6 Superoxide dismutase (SOD) and catalase (CAT) activity in murine peritoneal macrophages of different experimental groups

| Parameter | Superoxide dismutase (units/mg protein) | Catalase (mmol H₂O₂ decompose/min/mg protein) |
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| Control   | 34.102 ± 0.295                         | 4.802 ± 0.245                               |
| Nicotine  | 20.263 ± 0.426*                        | 1.812 ± 0.153*                             |
| 25.0 μg/ml ME-Og | 27.95 ± 0.773*             | 2.726 ± 0.209*                             |

Protein oxidation mediated cellular injury in macrophage, thus playing a protective role against oxidative immune cell damage preserving the cellular integrity.
**Materials and Methods**

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), standard reduced glutathione (GSH), NADPH Na₄, 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 and were of the highest grade available.

Isolation of the peritoneal macrophages and cell culture. Experiments were performed using Swiss male mice 6–8 weeks old, weighing 20–25 g. 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. 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 hrs injection of 2 ml of 4% starch according to our previous lab report by Kar Mahapatra et al.25 In brief, 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 penicillin and 50 μg/ml streptomycin for 24 h at 37°C in a humidified atmosphere of 5% CO₂–95% air in CO₂ 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%.25,42,43

Plant material and preparation of methanol extract of *O. gratissimum* (ME-Og). *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 Department of Botany, Vidyasagar University. The fresh aerial part of *O. gratissimum* was dried, blended and extracted with methanol (10:1). The mixture was filtered with Whatman filter paper (No. 1) and concentrated at 38°C by a rotary evaporator, then allowed to stand at room temperature overnight. This concentrated solution was then centrifuged at 2,000 xg for 10 min and supernatant was freeze dried to obtain the crude methanol extract.

Total flavonoid determination in ME-Og. Aluminum chloride colorimetric method was used for flavonoid determination in *O. gratissimum*.44 Plant methanol 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 1.0 M 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). The calibration curve was prepared by preparing quercetin solutions at concentrations 10 to 100 μg/ml in methanol.

**Table 5 Glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S transferase (GST) activity in murine peritoneal macrophages of different experimental groups**

| Parameter                  | Glutathione peroxidase (n mol NADPH consumed/min/mg protein) | Glutathione reductase (n mol NADPH consumed/min/mg protein) | Glutathione-S-transferase (n mol/min/mg protein) |
|----------------------------|-------------------------------------------------------------|-----------------------------------------------------------|--------------------------------------------------|
| Control                    | 23.802 ± 0.498                                              | 5.88 ± 0.414                                               | 3.318 ± 0.222                                    |
| Nicotine                   | 12.53 ± 0.77                                               | 1.356 ± 0.144                                               | 1.138 ± 0.101                                    |
| 25.0 μg/ml ME-Og           | 28.182 ± 0.641                                               | 7.111 ± 0.564                                               | 3.875 ± 0.145                                    |
| N + 1.0 μg/ml ME-Og        | 14.328 ± 0.762                                               | 1.973 ± 0.176                                               | 1.426 ± 0.164                                    |
| N + 5.0 μg/ml ME-Og        | 16.023 ± 0.934#                                             | 2.771 ± 0.157#                                             | 1.63 ± 0.129#                                   |
| N + 10.0 μg/ml ME-Og       | 18.565 ± 0.488*                                             | 4.296 ± 0.302*                                             | 1.835 ± 0.107*                                  |
| N + 25.0 μg/ml ME-Og       | 17.981 ± 0.871*                                             | 4.143 ± 0.359*                                             | 2.033 ± 0.128*                                  |

After the treatment schedule, activities of these three glutathione dependent antioxidant enzymes were measured spectrophotometrically. The results indicate that, GPx, GR and GST activity are significantly (p < 0.05) decreased in nicotine treated cells than control, which are significantly (p < 0.05) enhanced by supplementation of methanol extract of *O. gratissimum* (ME-Og). More over, these antioxidant enzymes activity are significantly (p < 0.05) enhanced in only ME-Og treated cells than cells in control group. So, ME-Og can boost up these glutathione dependent antioxidant enzymes and protect cell from nicotine toxicity. Values are expressed as mean ± SEM, n = 6. *indicates significant difference (p < 0.05) compared to nicotine treated group. # indicates significant difference (p < 0.05) compared to control group.

Total phenols determination in ME-Og. Total phenols were determined by Folin Ciocalteu reagent.45 A dilute ME-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 Na₂CO₃ (4 ml, 1.0 M). 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/L 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.

Cytotoxicity assay by MTT method. Cell cytotoxicity assay was performed by 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method according to Mosmann.46 Murine peritoneal macrophages were treated with ME-Og at concentrations ranging from 0.1 μg/ml to 100.0 μg/ml were further cultured in RPMI-1640 supplemented with 10% FBS for 24 h. Thereafter, the medium was replaced with fresh RPMI (without Phenol Red and FBS) containing 0.5 mg/ml of MTT. After additional 3 h incubation at 37°C, HCl-isopropanol solution was added to each culture plate. After 15 min of incubation at room temperature, absorbance of solubilized MTT formazan product was spectrophotometrically measured at 570 nm.
O. gratissimum protect nicotine-induced cellular damage

Group 1: Control i.e., culture media
Group 2: 10 mM Nicotine in culture media
Group 3: 25 μg ME-Og/ml culture media
Group 4: 10 mM Nicotine + 1 μg ME-Og/ml culture media
Group 5: 10 mM Nicotine + 5 μg ME-Og/ml culture media
Group 6: 10 mM Nicotine + 10 μg ME-Og/ml culture media
Group 7: 10 mM Nicotine + 25 μg ME-Og/ml culture media

After the treatment schedule, the treated cells are subjected to stain with Giemsa and morphological analysis has been done. The concentration of nicotine was selected according to our previous lab report. After the treatment schedule the cells were collected from the petridishes separately and centrifuged at 2,200 rpm for 10 min at 4°C. Then the supernatant was collected in separate micro centrifuge tube and the cells were washed twice with 50 mM PBS, pH 7.4. The pallets were lysed with hypotonic lysis buffer (10 mM TRIS, 1 mM EDTA and Titron X-100, pH 8.0) for 45 min at 37°C and then processed for the biochemical estimation. Intact cells were used for superoxide anion generation and NADPH oxidase activity.

Biochemical estimation. Assessment of superoxide anion (O₂⁻) generation. Superoxide anion generation was determined by a standard assay. 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 treatment schedule, and washing with PBS. Cytochrome-c reduction by generated superoxide was then determined by spectrophotometric absorbance at a 550 nm wavelength. Results are expressed n mol of cytochrome-c reduced/min, using extinction-coefficient 2.1 x 10⁴ M⁻¹ cm⁻¹.

NADPH oxidase activity. After the treatment schedule, the macrophages of different groups prewarmed in Krebs ringer buffer (KRB) with 10 mM glucose at 37°C for 3 min. PMA (0.1 μg/ml) prewarmed at 37°C 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 0.34 M sucrose. The cells were then lysed with hypotonic lysis buffer. Centrifugation was carried out at 800 xg 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 10 mM phosphate buffer (pH 7.2), 100 mM NaCl, 1 mM MgCl₂, 80 μM cytochrome c, 2 mM NaN₃ 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.

Myeloperoxidase (MPO) activity. 200 μl of cell lysate was reacted with 200 μl substrate (containing H₂O₂ and OPD) in 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 reading was taken at 492 nm in a spectrophotometer.

Determination of lipid peroxidation (MDA). Lipid peroxidation was estimated by the method of Ohkawa et al. 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 FeSO₄. After incubating the samples at 37°C 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°C for 45 min. After cooling, samples were centrifuged, and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using 1.53 x 10^5 M^-1 cm^-1 as extinction coefficient. The levels of lipid peroxidation were expressed in terms of μmol/mg protein.

**Protein carbonyls contents (PC).** Protein oxidation was monitored by measuring protein carbonyl contents by derivatization with 2, 4-dinitrophenyl hydrazine (DNPH). In general, cell lysate proteins in 50 mM potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2 N HCl). Blank samples were mixed with 2 N 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 6.0 N guanidine hydrochloride and absorbance was measured at 370 nm. Protein carbonyls content was expressed in terms of μmol/mg protein.

**Activity of super oxide dismutase (SOD).** SOD activity was determined from its ability to inhibit the auto-oxidation of pyrogalol according to Mestro Del and McDonald. The reaction mixture consisted of 50 mM Tris (hydroxymethyl) amino methane (pH 8.2), 1 mM diethylenetriamine penta acetic acid, and 20–50 μl of cell lysate. The reaction was initiated by addition of 0.2 mM pyrogalol, and the absorbance measured kinetically at 420 nm at 25°C for 3 min. SOD activity was expressed as unit/mg protein.

**Activity of catalase (CAT).** Catalase activity was measured in the cell lysate by the method of Luck. The final reaction volume of 3 ml contained 0.05 M Tris-buffer, 5 mM EDTA (pH 7.0), and 10 mM H₂O₂ (in 0.1 M potassium phosphate buffer, pH 7.0). About 50 μl aliquot of the cell 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 H₂O₂. The level of CAT was expressed in terms of m mol H₂O₂ consumed/min/mg protein.

**Determination of reduced glutathione (GSH).** Reduced glutathione estimation in the cell lysate was performed by the method of Moron et al. The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2,000 xg 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.

**Oxidized glutathione level (GSSG).** The oxidized glutathione level was measured after derevatization of GSH with 2-vinylpyridine according to the method of Griffith. In brief, with 0.5 ml cell lysate, 2 μl 2-vinylpyridine was added and incubates for 1 hr at 37°C. Then the mixture was deproteinized with 4% sulfosalicylic acid and centrifuged at 1,000 xg for 10 min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve.

**Redox ratio (GSH/GSSG).** Redox ratio was determined for all the seven groups by taking the ratio of reduced glutathione/oxidized glutathione.

**Activity of glutathione peroxidase (GPxs).** The GPxs activity was measured by the method of Paglia and Valentine. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U glutathione reductase and 1 mM reduced glutathione. The sample, after its addition, was allowed to equilibrate for 5 min at 25°C. The reaction was initiated by adding 0.1 ml of 2.5 mM H₂O₂. Absorbance at 340 nm was recorded for 5 min. Values were expressed as μmol of NADPH oxidized to NADP by using the extinction coefficient of 6.2 x 10³ M^-1 cm^-1 at 340 nm. The activity of GPxs was expressed in terms of μmol NADPH consumed/min/mg protein.

**Activity of glutathione reductase (GR).** The GR activity was measured by the method of Miwa. The tubes for enzyme assay were incubated at 37°C and contained 2.0 ml of 9 mM GSSG, 0.02 ml of 12 mM NADPH, Na₃, 2.68 ml of 1/15 M 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 n mol NADPH consumed/min/mg protein.

**Activity of glutathione-s-transferase (GST).** The activity of GST activity was measured by the method of Habig et al. The tubes of enzyme assay were incubated at 25°C and contained 2.85 ml of 0.1 M potassium phosphate (pH 6.5) containing 1 mM of GSH, 0.05 ml of 60 mM 1-chloro-2, 4-dinitrobengene and 0.1 ml cell lysate. The activity of this enzyme was determined by monitoring the increase in absorbance at 340 nm.

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

**Statistical analysis.** The data were expressed as mean ± standard error, n = 6. Comparisons of the means of control, nicotine and nicotine with different concentration of ME-Og treated group were made by two-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA) with multiple comparison t-tests, p < 0.05 as a limit of significance.

**References**

1. Gupta SK, Prakash J, Srivastava S. Validation of traditional claim of rulsi, Ocimum sanctum Linn. as a medicinal plant. J Ind Exp Biol 2002; 40:765-73.
2. Ilori M, Shetelu AO, Omobimbin EA, Adenyee AA. Antioxidant activities of Ocimum gratissimum (Lamiaceae). J Diarrhoeal Dis Res 1996; 14:283-5.
3. Onajobi FD. Smooth muscle contracting lipidsoluble principles in chromatographic fractions of Ocimum gratissimum. J Ethnopharmacol 1986; 18:3-11.
4. Obasekiri-Ebor EE, Odukoya K, Teskepally H, Mitscher LA, Shankel DM. Antimutagenic activity of extracts of leaves of four common edible vegetable plants in Nigeria (West Africa). Mutat Res 1993; 302:189-17.
5. Nakamura CV, Ueda-Nakamura T, Bando E, Mitscher LA, Shankel DM. Antimutagenic activity of extracts of leaves of four common edible vegetable plants in Nigeria (West Africa). Mutat Res 1993; 302:189-17.
6. Orafidiya LO, Oyedele AO, Shittu AO, Elujoba AA. The formulation of an effective topical antibacterial product containing Ocimum gratissimum leaf essential oil. Mem Inst Oswaldo Cruz 1999; 94:675-8.
7. Orafidiya LO, Oyedele AO, Shittu AO, Elujoba AA. The formulation of an effective topical antibacterial product containing Ocimum gratissimum leaf essential oil. Mem Inst Oswaldo Cruz 1999; 94:675-8.
8. Narayana MO, Okarfi JR. Preliminary studies of the antifungal activities of some medical plants against Basidiobolus and some other pathogenic fungi. Mycoses 1995; 38:191-5.
8. Nakamura CV, Ishida K, Faccin LG, Filho BPD, Cortez DAG, Rozenval S, et al. In vitro activity of essential oil from Ocimum gratissimum L. against four Candida species. Research in Microbiology 2004; 155:579-86.

9. Lemos JA, Passos XS, Ferna OPL, Paula JR, Ferri PH, Souza LKH, et al. Antifungal activity from Ocimum gratissimum L. towards Cryptococcus neoformans. Mem Inst Oswaldo Cruz, Rio de Janeiro 2005; 100:55-8.

10. Sartorio A, Machado ALM, Delamurina C, Figueira GM, Duarte-MCT, Rehder VLG. Composition and antimicrobial activity of essential oils from aroatic plants used in Brazil. Brazilian Journal of Microbiology 2004; 35:275-80.

11. Pessoa LM, Morais SM, Bevilaqua CML, Luciano JHS. Antihelminthic activity of essential oil of Ocimum gratissimum Linn. and eugenol against Haemonchus contortus. Veterinary Parasitology 2002; 109:59-63.

12. Silva MR, Oliveira JG Jr, Fernandes OF, Passos XS, Costa CR, Souza LK, et al. Antifungal activity of Ocimum gratissimum towards dermatomyces. Mycoses 2005; 48:172-5.

13. Prakash J, Gupta SK, Singh N, Kochupilla V, Gupta YK. Antiplatelet and chemo preventive activity of Ocimum sanctum Linn. Int J Med Biol Environ 1999; 27:165.

14. Nangia-Makker P, Tait T, Shekhtar MPV, Palomino E, Hogan V, Piechocki MP, et al. Inhibition of breast tumor growth and angiogenesis by a medicinal herb: Ocimum gratissimum. Int J Cancer 2007; 121:884-94.

15. Schievelbein H. Nicotine, resorption and fate. Pharmac Ther 1982; 18:233-48.

16. Doolittle DJ, Wingar R, Lee CK, Caldwell WS, Hayes AW, deBertHJ. The genotoxic potential of nicotine and its major metabolites. Mutat Res 1995; 344:95-102.

17. Yildiz D, Eckel N, Armstrong DW. Nicotine enantiomers and oxidative stress. Toxicology 1998; 130:55-65.

18. Kovicic P, Cooksy A. Iminium metabolite mechanism for nicotine toxicity and addiction: Oxidative stress and electron transfer. Medical Hypotheses 2005; 64:104-11.

19. Das S, Neogy S, Goutam N, Roy S. In vitro nicotine induced superoxide mediated DNA fragmentation in lymphocytes: Protective role of Andrographis paniculata Nees. Toxicol In Vitro 2005; 23:99-07.

20. Limpijumnong S, Ravri SI. Protection of lipid peroxidation and carbonyl formation in proteins by capsaicin in human erythrocytes subjected to oxidative stress. Phytother Res 2006; 20:303-6.

21. Muthukumaran S, Sudheer AR, Menon VP, Nalini N. Protective effect of quercetin on nicotine-induced prooxidant and antioxidant imbalance and DNA damage in Wistar rats. Toxicol In Vitro 2008; 22:207-15.

22. Neogy S, Das S, Kar Mahaparra S, Mandal N, Roy S. Amdelioratory effects of Andrographis paniculata Nees on liver, kidney, heart, lung and spleen during nicotine induced oxidative stress. Environ Toxicol Pharmacol 2008; 25:321-8.

23. Sudheer AR, Muthukumaran S, Kalpana C, Srinivasan M, Menon VP. Protective effect of fenical on nicotine-induced DNA damage and cellular changes in cultured rat peripheral blood lymphocytes: A comparison with N-acetylcysteine. Toxicol In Vivo 2007a; 21:576-5.

24. Sudheer AR, Muthukumaran S, Devipriya N, Menon VP. Ellagic acid, a natural polyphenol protects rat peripheral blood lymphocytes against nicotine-induced cellular and DNA damage in vitro: With the comparison of N-acetylcysteine. Toxicol In Vitro 2007b; 230:11-21.

25. Kar Mahaparra S, Das S, Bhattacharjee S, Goutam N, Majumdar S, Roy S. In vitro nicotine-induced oxidative stress in mice peritoneal macrophages: a dose-dependent approach. Toxicology and Mechanisms and Methods 2009; 19:100-8.

26. Das S, Kar Mahaparra S, Goutam N, Das A, Roy S. Oxidative stress in lymphocytes, neutrophils and serum of oral cavity cancer patients: modulatory array of L-glutamine. Support Care Cancer 2007; 15:399-409.

27. Ursuane ER. Macrophages, antigen-presenting cells, and the phenomena of antigen handling and presentation. In Paul WE, (Ed.), Fundamental Immunology, Raven Press, New York 1989; 95-115.

28. Victor MV, De La Fuente M. Immune cells redox status of aged persons: overview of present evidence. J Am Clin Nutr 1995; 62:1462-76.