[Oxidative Medicine and Cellular Longevity 2:4, 222-230; September/October 2009]; ©2009 Landes Bioscience

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

Santanu Kar Mahapatra, Subhankari Prasad Chakraborty, Subhasis Das and Somenath Roy*

Immunology and Microbiology Laboratory; Department of Human Physiology with Community Health; Vidyasagar University; Midnapore, West Bengal India

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, thiobutiric acid; TBARS, thiobutiric 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 generation, 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.

*Correspondence to: Somenath Roy; Immunology and Microbiology Laboratory; Department of Human Physiology with Community Health; Vidyasagar University; Midnapore, West Bengal 721 102 India; Tel./Fax: +91.3222.275329; Email: somenathroy@hotmail.com

Submitted: 04/23/09; Revised: 05/13/09; Accepted: 05/13/09

Previously published online as an *Oxidative Medicine and Cellular Longevity* E-publication:
http://www.landesbioscience.com/journals/oximed/article/9000

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.[3,4] This medicinal plant has also potential role as antibacterial, antifungal, antimicrobial, anthelmintic, and in vitro antidermatophytic agent.[5-12] The aqueous leaf extract and seed oil showed anti-proliferative and chemo-preventive activity on HeLa cells.[13] Nangia-Makker et al. reported that, aqueous extract of *O. gratissimum* leaves inhibits tumor growth and angiogenesis by affecting tumor cell proliferation, migration, morphogenesis, stromal apoptosis and induction of inducible cyclooxygenase (COX-2).[14]

Nicotine is an alkaloid which is composed of a pyridine and a pyrrolidine ring. It is found in the plant kingdom throughout a wide range of families.[15,16] 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).[17] These ROS in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation, protein oxidation and DNA damage.[18,19] Lipid peroxidation is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerization of polysaccharide, as well as protein cross-linking and fragmentation.[20] 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.[21,22] In vitro experiments by many researchers were also shown that, nicotine severely damages the DNA and imbalance the prooxidant/antioxidant status in lymphocytes.[19,23,24] 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.[25] 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.[26]

Macrophages are ubiquitous mononuclear phagocytes in mammalian tissues. The peritoneal macrophages are representative of other macrophage populations,[27] and also easily available in mice in greater amounts than blood phagocytes (monocytes or neutrophils). The immune cell functions are specially linked to ROS generation and are strongly influenced by the redox potential. Therefore the oxidant/antioxidant balance is an important determinant of immune cell activity. The antioxidant levels in immune cells play a pivotal role in protecting them against oxidative stress and therefore preserving their adequate function. In our previous lab report, it was clearly established that nicotine can damage the murine peritoneal macrophages.[25] 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.

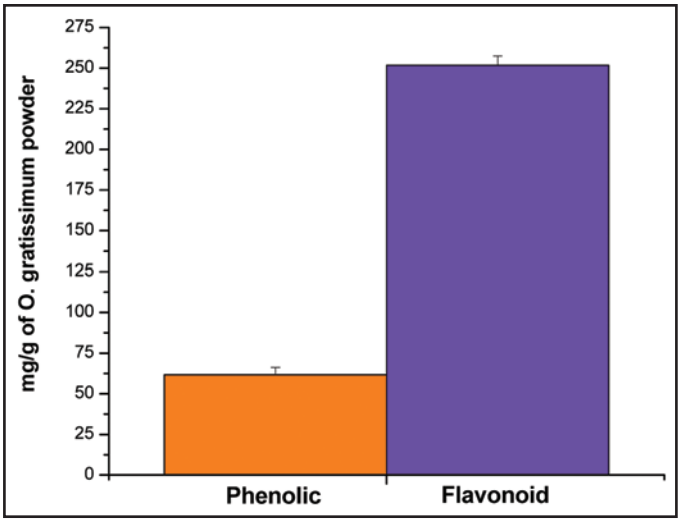

Figure 1. Phenolic and flavonoid content in methanol extract of *O. gratissimum* (ME-Og) were determined in UV-Vis spectrophotomer using quercetin (in case of flavonoid), and gallic acid (in case of phenolics) as the standard. The results indicate that flavonoid is higher amount in ME-Og than phenolic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

Results

Total phlenolic 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 ($O_2^{\cdot-}$) 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ generation significantly ($p < 0.05$), when compared to nicotine treated group. Where, 1 μg/ml ME-Og supplementation could decreased

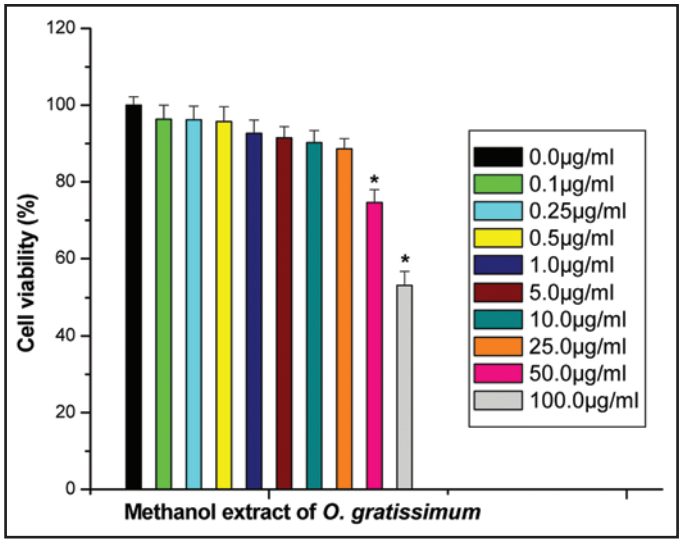

Figure 2. Dose-response curve of the effect of methanol extract of *O. gratissimum* (ME-Og) on cell cytoxicity as well as viability of peritoneal macrophage. Murine peritoneal macrophages cultured in cell culture media, were subjected to ME-Og treatment at specified doses (0.1, 0.25, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0 µg/ml). After 24 hrs of incubation, a cell viability assay was performed using MTT method. The results indicate that, up to 25 µg/ml of ME-Og does not producing any significant reduction of cell viability, where as 50 µg/ml, and 100 µg/ml ME-Og treatment can significantly reduce the cell viability of macrophage. The experiment was repeated three times, yielding similar results and data are shown as mean ± SEM. * indicates significant differences compared with untreated control group at the level of $p < 0.05$.

the $O_2^{\cdot-}$ generation 7.14% and also decreased NADPH oxidase activity 6.12%, there 25 µg/ml ME-Og exerted maximum protective effect, and reduced the $O_2^{\cdot-}$ generation 26.05% and NADPH oxidase activity 31.93% compared with only nicotine treated group (Table 1).

Myeloperoxidase (MPO) activity. MPO activity in murine peritoneal macrophages in different group is shown in Table 1. MPO is an important enzyme to produce hypochlorus acid (HOCl) in cellular system that leads to oxidative damage. So, it is an important determinant to establish the free radical generation in peritoneal macrophage. MPO activity is significantly ($p < 0.05$) increased in nicotine treated group by 116.71% as compared to the control group. ME-Og (1 µg/ml, 5 µg/ml, 10 µg/ml and 25 µg/ml) decrease the excess MPO activity significantly ($p < 0.05$) compared to their nicotine treated group. Out of the four 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).

Glutathione level and redox status. Glutathione is an important antioxidant in cellular system. So, to understand glutathione level, we have measured both reduced and oxidized form of glutathione. The 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). But only ME-Og treatment (25 µg/ml) slightly increased GSSG level (2.650%) 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 is the most effective dose to play a protective role against the nicotine toxicity. Only ME-Og treatment (25 µg/ml) could enhance the SOD, CAT, GPx, GR, GST activity (Tables 4 and 5).

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

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 $O_2^{\cdot -}$.[29] This $O_2^{\cdot -}$ 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 $O_2^{\cdot -}$ was found in this present study. Thus *O. gratissimum* protect the immune cell through reducing the NADPH oxidase activity and excess $O_2^{\cdot -}$ generation. In the presence of macrophage derived MPO, ROS generates hypochlorus acid (HOCl) 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

Table 1 The status of super oxide anion ($O_2^{\cdot -}$) generation, NADPH oxidase activity and myeloperoxidase (MPO) activity in murine peritoneal macrophages of different experimental groups

Parameter	Superoxide anion (n mol/min/ 2 x 10^6 cell)	NADPH oxidase activity (n mol/min/ 2 x 10^6 cell)	Myeloperoxidase activity (unit/mg protein)
Control	0.553 ± 0.021	0.482 ± 0.028	0.73 ± 0.061
Nicotine	1.121 ± 0.034*	1.046 ± 0.032*	1.582 ± 0.073*
25.0 μg/ml ME-Og	0.426 ± 0.019*	0.428 ± 0.026	0.68 ± 0.041
N + 1.0 μg/ml ME-Og	1.041 ± 0.035	0.982 ± 0.055	1.324 ± 0.063#
N + 5.0 μg/ml ME-Og	0.965 ± 0.021#	0.906 ± 0.022#	1.221 ± 0.044#
N + 10.0 μg/ml ME-Og	0.847± 0.029#	0.739 ± 0.033#	1.18 ± 0.042#
N + 25.0 μg/ml ME-Og	0.829 ± 0.03#	0.712 ± 0.054#	1.083 ± 0.036#

After the treatment schedule, these parameters were estimated using UV-Vis spectrophotometer. All of these three parameters were significantly ($p < 0.05$) increased in nicotine treated macrophages compared with control and slightly decreased in only ME-Og treated macrophages compared with control group of cells. Beside that, supplementation of ME-Og with nicotine concentration dependently decreased the excess $O_2^{\cdot -}$ generation, NADPH oxidase activity, and MPO activity. So, ME-Og may play a protective role against nicotine toxicity in murine peritoneal macrophages through decreasing the free radical generation. 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 2 Lipid peroxidation level and protein carbonyls level in murine peritoneal macrophages of different experimental groups

Parameter	Malondialdehyde (μ mol/mg protein)	Protein carbonyl (μ mol/mg protein)
Control	0.178 ± 0.017	5.115 ± 0.09
Nicotine	0.535 ± 0.027*	12.048 ± 0.261*
25.0 μg/ml ME-Og	0.203 ± 0.012	5.034 ± 0.277
N + 1.0 μg/ml ME-Og	0.497 ± 0.013	11.886 ± 0.408
N + 5.0 μg/ml ME-Og	0.466 ± 0.017#	9.42 ± 0.312#
N + 10.0 μg/ml ME-Og	0.335 ± 0.015#	9.056 ± 0.211#
N + 25.0 μg/ml ME-Og	0.322 ± 0.01#	9.14 ± 0.365#

After the treatment schedule, the lipid peroxidation in terms of malondialdehyde (MDA) and protein oxidation in terms of protein carbonyl (PC) formation were measured spectrophotometrically. The results indicate that, MDA and PC levels are significantly ($p < 0.05$) increased in nicotine treated group than control group; which are significantly ($p < 0.05$) reduced by supplementation of methanol extract of *O. gratissimum* (ME-Og). More over, there is no significant alteration of MDA or PC level, when cells are treated with only ME-Og. So, ME-Og is protecting lipid and protein in murine peritoneal macrophages against 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 3 **Reduced glutathione (GSH), oxidized glutathione (GSSG) and redox ratio (GSH/GSSG) in murine peritoneal macrophages of different experimental groups**

Parameter	Reduced glutathione (μ gm/mg protein)	Oxidized glutathione (μ 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
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#

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 ($p < 0.05$) decreased, but GSSG level is significantly ($p < 0.05$) increased in nicotine treated cells than control; which are significantly ($p < 0.05$) 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.

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 H_2O_2 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	36.287 ± 0.578*	5.13 ± 0.126
N + 1.0 μg/ml ME-Og	22.317 ± 0.354	1.922 ± 0.11
N + 5.0 μg/ml ME-Og	27.14 ± 0.533#	2.103 ± 0.125
N + 10.0 μg/ml ME-Og	27.563 ± 0.551#	2.688 ± 0.141#
N + 25.0 μg/ml ME-Og	27.95 ± 0.773#	2.726 ± 0.209#

After the treatment schedule, SOD and CAT activity were measured spectrophotometrically. The results indicate that, SOD and CAT are significantly ($p < 0.05$) decreased in nicotine treated group than control group; which are significantly ($p < 0.05$) 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.

protein oxidation mediated cellular injury in macrophage, thus playing a protective role against oxidative immune cell damage preserving the cellular integrity.

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.[36] 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[37] 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.[38] Depletion in the activities of SOD, CAT, GPx and GR 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[39] 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 (GP_X, 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, NADPH oxidase activity, 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.[40] Phenolic compounds act as free radical scavengers by virtue of their hydrogen donating ability.[41] Therefore, presence of phenolic compound in ME-Og may boost up the antioxidant property of *O. gratissimum.*

In conclusion, the study described here, demonstrate the methanol extract of *O. gratissimum* protects the murine peritoneal macrophages from nicotine toxicity by decreasing free radical generation, lipid and protein damage, and also by increasing the antioxidant status. Hence, the ME-Og can be used as a potent free radical scavenger antioxidative product and can be used as a potential therapeutic agent against nicotine toxicity.

Materials and Methods

Chemicals and reagents. Hydrogen tartarate salt of nicotine, phorbol mirested aceted (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 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 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 gentamycin, 50 μg/ml penicillin and 50 μg/ml streptomycin for 24 h at 37°C 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%.[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 Deptartment 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 x*g* 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 (m 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 control group. #indicates significant difference ($p < 0.05$) compared to nicotine treated 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 Na_2CO_3 (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-isopropanolic 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.

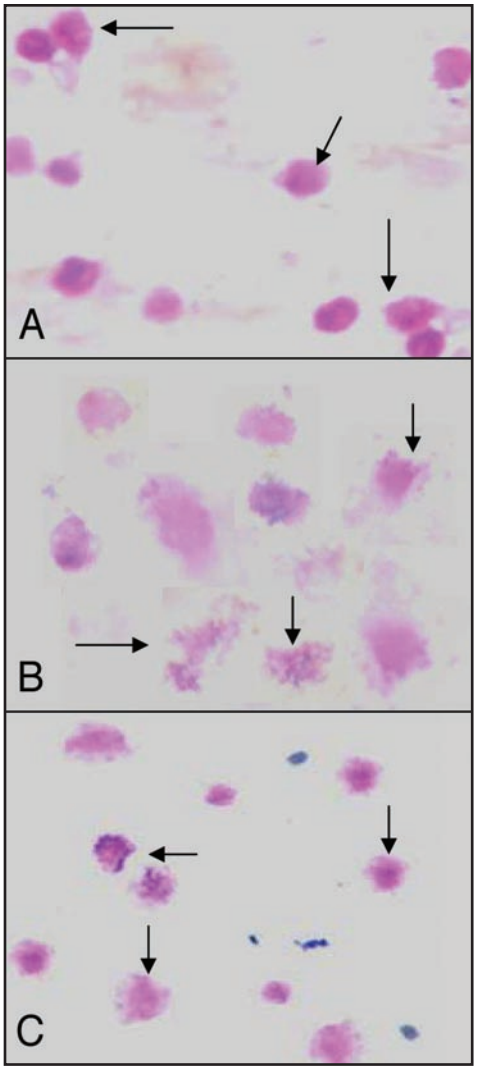

Figure 3. Morphology of normal, nicotine (10 mM) treated and Nicotine (10 mM) + methanol extract of *O. gratissimum* (ME-Og) (25 μg/ml) treated murine peritoneal macrophages. After the treatment, the murine peritoneal macrophages are stained with Giemsa and observed under phage contrast microscope at 1,000X magnification. (A) Normal peritoneal macrophages. (B) Peritoneal macrophages are damaged due to nicotine treatment. (C) Supplementation of ME-Og with nicotine protects the peritoneal macrophages from nicotine induced cellular damage.

Preparation of nicotine. Hydrogen tartarate salt of nicotine, obtained from Sigma was dissolved in normal saline (0.9% NaCl) to get the required concentration. Then pH of the nicotine solution was adjusted to 7.4 by NaOH.[25]

Experimental design and sample preparation. The peritoneal macrophages were divided into seven groups. Each group contained six petridishes (4 x 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 gentamycin, 50 μg/ml penicillin and 50 μg/ml streptomycin at 37°C in a 95% air/5% CO_2 atmosphere in CO_2 incubator.

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

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.[25] 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.[47] Intact cells were used for superoxide anion generation and NADPH oxidase activity.

Biochemical estimation. Assessment of superoxide anion ($O_2^{\cdot-}$) generation. Superoxide anion generation was determined by a standard assay.[48] 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 10^4 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 $MgCl_2$, 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.[49]

Myeloperoxidase (MPO) activity. 200 μl of cell lysate was reacted with 200 μl substrate (containing H_2O_2 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.[50]

Determination of lipid peroxidation (MDA). Lipid peroxidation was estimated by the method of Ohkawa et al. in cell lysate.[51] 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°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 1.53×10^5 M^{-1} cm^{-1} as extinction coefficient. The levels of lipid peroxidation were expressed in terms of n mol/mg protein.

Protein carbonyls contents (PC). Protein oxidation was monitored by measuring protein carbonyl contents by derivatization with 2, 4-dinitrophenyl hydrazine (DNPH).[52] 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 μ mol/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.[53] The reaction mixture considered 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.[54] The final reaction volume of 3 ml contained 0.05 M Tris-buffer, 5 mM EDTA (pH 7.0), and 10 mM H_2O_2 (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 H_2O_2. The level of CAT was expressed in terms of m mol H_2O_2 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.[55] 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-vinylpyidine according to the method of Griffith.[56] In brief, with 0.5 ml cell lysate, 2 μl 2-vinylpyidine was added and incubates for 1 hr at 37°C. Then the mixture was deprotenized 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 (GPx). The GPx activity was measured by the method of Paglia and Valentine.[57] 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 H_2O_2. Absorbance at 340 nm was recorded for 5 min. Values were expressed as n mol 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 n mol NADPH consumed/min/mg protein.

Activity of glutathione reductase (GR). The GR activity was measured by the method of Miwa.[58] 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, Na_4, 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.[59] 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.[60]

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 tulsi, *Ocimum sanctum* Linn. as a medicinal plant. Ind J Exp Biol 2002; 40:765-73.
2. Ilori M, Sheteolu AO, Omonibgehin EA, Adeneye AA. Antidiarrhoeal activities of *Ocimum gratissimum* (Lamiaceae). J Diarrhoeal Dis Res 1996; 14:283-5.
3. Onajobi FD. Smoth muscle contracting lipidicsoluble principles in chromatographic fractions of *Ocimum gratissimum.* J Ethnopharmacol 1986; 18:3-11.
4. Obaseiki-Ebor EE, Odukoya K, Telikepally H, Mitscher LA, Shankel DM. Antimutagenic activity of extracts of leaves of four common edible vegetable plants in Nigeria (West Africa). Mutat Res 1993; 302:109-17.
5. Nakamura CV, Ueda-Nakamura T, Bando E, Melo AF, Cortez DA, Dias Filho BP. Antibacterial activity of *Ocimum gratissimum* L. essential oil. Mem Inst Oswaldo Cruz 1999; 94:675-8.
6. Orafidiya LO, Oyedele AO, Shittu AO, Elujoba AA. The formulation of an effective topical antibacterial product containing *Ocimum gratissimum* leaf essential oil. International Journal of Pharmaceutics 2001; 224:177-83.
7. Nwosu MO, Okafor JI. 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 LC, Filho BPD, Cortez DAG, Rozental 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 OFL, 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. Sartoratto A, Machado ALM, Delarmelina C, Figueira GM, Duarte MCT, Rehder VLG. Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. Brazilian Journal of Microbiology 2004; 35:275-80.
11. Pessoa LM, Morais SM, Bevilaqua CML, Luciano JHS. Anthelmintic 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 dermatophytes. Mycoses 2005; 48:172-5.
13. Prakash J, Gupta SK, Singh N, Kochupillai V, Gupta YK. Antiproliferative and chemo preventive activity of *Ocimum sanctum* Linn. Int J Med Biol Environ 1999; 27:165.
14. Nangia-Makker P, Tait L, Shekhar 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. Pharm Ther 1982; 18:233-48.
16. Doolittle DJ, Winegar R, Lee CK, Caldwell WS, Hayes AW, deBethizy JD. The genotoxic potential of nicotine and its major metabolites. Mutat Res 1995; 344:95-102.
17. Yildiz D, Ercal N, Armstrong DW. Nicotine enantiomers and oxidative stress. Toxicology 1998; 130:155-65.
18. Kovacic 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, Gautam N, Roy S. In vitro nicotine induced superoxide mediated DNA fragmentation in lymphocytes: Protective role of *Andrographis paniculata* Nees. Toxicol In Vitro 2009; 23:90-8.
20. Luqman S, Rizvi 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 2008; 243:207-15.
22. Neogy S, Das S, Kar Mahapatra S, Mandal N, Roy S. Amelioratory effect of *Andrographis paniculata* Nees on liver, kidney, heart, lung and spleen during nicotine induced oxidative stress. Env Toxicol Pharmacol 2008; 25:321-8.
23. Sudheer AR, Muthukumaran S, Kalpana C, Srinivasan M, Menon VP. Protective effect of ferulic acid on nicotine-induced DNA damage and cellular changes in cultured rat peripheral blood lymphocytes: A comparison with *N*-acetylcysteine. Toxicol In Vitro 2007a; 21:576-85.
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 2007b; 230:11-21.
25. Kar Mahapatra S, Das S, Bhattacharjee S, Gautam N, Majumdar S, Roy S. In vitro nicotine-induced oxidative stress in mice peritoneal macrophages: a dose-dependent approach. Toxicology Mechanisms and Methods 2009; 19:100-8.
26. Das S, Kar Mahapatra S, Gautam 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; 15:1399-409.
27. Unanue 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 state from mice with endotoxin-induced oxidative stress. Involvement of NFκB Stress. Free Radical Res 2002; 36:33-45.
29. Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. Arch Biochem Biophys 2002; 397:342-4.
30. Sullivan GW, Sarembock IJ, Linden J. The role of inflammation in vascular diseases. J Leuk Biol 2000; 67:591-602.
31. Suleyman H, Gumustakine K, Taysi S, Keles S, Oztasan N, Aktas O, et al. Beneficial effect of *Hippophase rhamnoides L* on nicotine induced oxidative stress in rat blood compared with vitamin E. Biol Pharm Bull 2002; 25:1133-6.
32. Wetscher GJ, Bagchi M, Bagchi D, Perdikis G, Hinder PR, Giaser K, Hinder RA. Free radicals production in nicotine-treated pancreatic tissue. Free Radical Biol Med 1995; 18:877-82.
33. Helen A, Krishnakumar K, Vijayammal PL, Augusti KT. Antioxidant effect of onion oil (*Allium cepa* Linn) on the damages induced by nicotine in rats as compared to alpha-tocopherol. Toxicol Lett 2000; 116:61-8.
34. Kalpana C, Menon VP. Modulatory effects of curcumin on lipid peroxidation and antioxidant status during nicotine-induced toxicity. Pol J Pharmacol 2004; 56:581-6.
35. Meydani SN, Wu D, Santos MS, Hayek MG. Antioxidants and immune response in aged persons: overview of present evidence. J Am Clin Nutr 1995; 62:1462-76.
36. Romao PR, Tovar J, Fonseca SG, Moraes RH, Cruz AK, Hothersall JS, et al. Glutathione and the redox control system trypanothione/trypanothione reductase are involved in the protection of Leishmania sp. against nitrosothiol-induced cytotoxicity. Braz J Med Biol Res 2006; 39:355-63.
37. Sarkar S, Yadav P, Trivedi R, Bansal AK, Bhatnagar D. Cadmium-induced lipid peroxidation and the status of the antioxidant system in rat tissues. J Elem Med 1995; 9:144-7.
38. Ray G, Husain SA. Oxidants, antioxidants and carcinogenesis. Ind J Exp Biol 2002; 42:1213-32.
39. Hemachand T, Gopalakrishnan B, Salunke DM, Totey SM, Shaha C. Sperm plasma-membrane-associated glutathione S-transferases as gamete recognition molecules. J Cell Sci 2002; 115:2053-65.
40. Rohrdanz E, OhleTran-Thi RS, Tran-Thi QH, Kahl R. The phytoestrogen daidzein affects the antioxidant enzyme system of rat hepatoma H4IIE cells. J Nutri 2002; 132:370-5.
41. Laranjinha JAN, Almeida LM, Madeira VM. Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low density lipoprotein peroxidation. Biochem Pharmacol 1994; 48:487-94.
42. Azuma Y, Ohura K. Comparison of the effect of lidocaineepinephrine and prilocaine-felypressine to alter macrophage functions. Int Immunopharmacol 2001; 1:911-23.
43. Oliveros LB, Videla AM, Gimenez MS. Effect of dietary fat saturation on lipid metabolism, arachidonic acid turnover and peritoneal macrophage oxidative stress in mice. Braz J Med Biol Res 2004; 37:311-20.
44. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Analysis 2002; 10:178-82.
45. McDonald S, Prenzler PD, Autolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts. Food Chemistry 2001; 73:73-84.
46. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55-63.
47. Sandhu SK, Kaur G. Alterations in oxidative stress scavenger system in aging rat brain and lymphocytes. Biogerontology 2002; 3:161-73.
48. Johnston RB Jr, Godzik CA, Cohn ZA. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. J Exp Med 1978; 148:115-27.
49. Heyneman RA, Vercauteren RE. Activation of a NADPH oxidase from horse polymorphonuclear leukocytes in a cell-free system. J Leukoc Biol 1984; 36:751-9.
50. Bos AR, Weaver R, Ross D. Characterization and quantification of the peroxidase in human neutrophils. Biochem Biophys Acta 1990; 525:4133-41.
51. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95:351-8.
52. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assay for determination of oxidatively modified proteins. Methods Enzymol 1994; 233:346-57.
53. Mestro Del RF, McDonald W. Oxidative enzymes in tissue homogenates. In Greenwald RA, (Ed.), *CRC handbook of methods for oxygen radical research*, CRC Press, Boca Raton FL 1986; 291-6.
54. Luck H. Catalase. In Bergmeyer HW, (Ed.), *Methods of Enzymatic Analysis*, Section 3, Academic Press, New York 1963; 885-94.
55. Moron MS, Kepierre JW, Mannervick B. Levels of glutathione reductase and glutathione-S-transferase activities in rat lung and liver. Biochim Biophys Acta 1979; 582:67-8.
56. Griffith OW. Determination of glutathione and glutathione sulfide using glutathione reductase and 2-Vinyl pyridine. Anal Biochem 1980; 106:207-12.
57. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70:158-69.
58. Miwa S. Hematology. Mod Med Technol 1972; 3:306-10.
59. Habig WH, Pabst MJ, Jakoby WB. Glutathione-S-transferases. The first enzymatic step in mercapyuric acid formation. J Biol Chem 1974; 249:7130-9.
60. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol Reagent. J Biol Chem 1951; 193:255-75.