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The possible protective effect of Spirulina platensis extract against Nicotine Induced-Lung Toxicity in Rats

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Abstract
Background: *Nicotine, a major component of cigarette smoke, plays an important role in the development of cardiovascular disease and lung cancer in smokers. The aim of the present work was to investigate protective activity of Spirulina platensis (SP) extract against lung toxicity induced by nicotine in adult rats.* ***Materials and Methods:*** *Thirty six adult albino rats weighing around 150 ±10 g were used for the evaluation of lung protective activity of SP (3% and 6%) against nicotine-induced lung toxicity in rats.* ***Results:*** *The daily oral administration of the SP (3% and 6%) for 30 days to rats treated with nicotine (2.5 mg/kg.b.w.) resulted in a significant improve in plasma cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol as well as serum tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and growth factor (TGF)-β1 in nicotine treated groups rats. On the other hand, oral administration of SP (3% and 6%) elevated the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and total protein kinase-1 (Akt-1) as well as reduced the level malondialdehyde (MDA) in lung rats treated with nicotine. In addition, SP reduced the expression of lung inducible nitric oxide synthase (iNOS) and mitogen-activated protein kinase (p38-MAPK) levels as compared to nicotine treated control group. Also, SP (3% and 6%) almost normalized these effects in the histoarchitecture of the lung. Conclusion: The obtained biochemical, molecular biology and histological results of this study proved the lung protective activity of SP (3% and 6%) against nicotine induced lung toxicity in rats.*

Key words: *Spirulina platensis, nicotine, lung, nanoparticles oxidative stress biomarkers, iNOS and p38-MAPK.*

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I. Introduction

Algae have developed several mechanisms to alleviate the harmful effects of ROS, including non-enzymatic antioxidants, such as chlorophyll [1], carotenoids [2] phycobiliprotein [3] phenolics [4] and enzymatic antioxidants [5, 6]. Spirulina platensis (SP) is a multicellular, filamentous cyanobacterium [7]. It grows particularly in alkaline, brackish, and marine water [8]. SP contains omega-3- and omega-6-polyunsaturated fatty acids, phycocyamin and other phytochemicals [9] in addition to several enzymatic and non-enzymatic antioxidant defense systems [10]. Both systems provide adequate protection against effects of reactive oxygen species under stress condition [11] by over productions of non-enzymatic antioxidants, cartenoids, tecopherols and ascorbic acid, glutathione and chlorophyll derivatives [12] and the primary scavenging enzymatic defense system which include superoxide dismutase, catalase and glutathione peroxidase [13]. Therapeutically, SP can be used for several medical conditions such as allergies, ulcers, anemia, heavy–metals and radiation poisoning [14], anti-nephrotoxic [15], antihepatotoxic and in experimental Parkinson's [16]. SP became widely known after its usage successfully by National Aeronautic and Space Administration (NASA) as a dietary supplement for astronauts on space missions. It has the ability to modulate immune functions and exhibits anti-inflammatory properties by inhibiting the release of histamine from mast cells. Many studies suggested that these algae may also improve several diseased conditions and may even have antiviral effect [17]. It has been suggested that the ability of SP to inhibit carcinogenesis is due to its antioxidant properties that protect tissue from cell damage [18]. The unique polysaccharide of SP enhances cell nucleus enzyme activity and potentiates the process of DNA repair [19]. SP reduced both chromosomal damage and lipid peroxidation induced by cyclophosphamide cisplatin and urethane in mice significantly [20].

As an extension of interested research program to evaluate the medicinal importance of natural products [20-23], this work aimed to evaluate the therapeutic potential of Spirulina platensis (SP) extract on rat lung toxicity induced by nicotine.

II. Materials and Methods

Drugs

Spirulina platensis water extract Powder (100%), was purchased from ZAZZEE NATRUALS, USA. All other chemicals used in this study were of the analytical grade.

Animals

Thirty six male albino rats weighing around 150±10 g were obtained from animal house of Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. They were housed in plastic cages with six animals in each with stainless steel covers at the National Cancer Institute Animal House. The animals were maintained at a temperature of 22±1°C and a humidity of 55–60% in a light-controlled room. The animals were kept for 1 week to acclimatize and provided with standard diet and water ad libitum.

Experimental setup

This experiment was carried out to examine the protective effect of SP against nicotine-induced lung toxicity. This experiment was conducted in accordance with guidelines established by the Animal Care and Use Committee of October 6th University. Adult albino rats were divided into six groups with six animals in each as following:

Group I (Normal control): received 3 mL of distilled water, orally for 30 days
Group II (3% SP group): included the rats received 3% SP mixed with diet [24].
Group III (6% SP group): included the rats received 6% SP mixed with diet [24].
Group IV (Nicotine group): Subcutaneous injection of 2.5 mg/kg b.w. nicotine [25] in tween 80, 1%, for 30 days
Group V (SP 3%+ Nicotine group): This group included rats with acute lung toxicity induced by nicotine and received treatment with 3% SP mixed with diet.
Group VI (SP 6%+ Nicotine group): This group included rats with acute lung toxicity induced by nicotine and received treatment with 6% SP mixed with diet.

Biochemical assays

Blood samples were withdrawn from the retro-orbital vein of each fasted animal. Blood was collected using sodium fluoride as anticoagulant, centrifuged, and plasma was used freshly for estimation of triglyceride, total cholesterol and HDL-C were determined [26, 27]. Plasma LDL-cholesterol level was calculated from Falholt et al [28] formula.

Plasma tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), TGF-β1 and total Akt-1 level were performed using a series of ELISA kits according to the manufacturer's protocol (Wuhan Boster Biological Technology, Ltd., Wuhan, China).. The lung superoxide dismutase assay kit utilizes a tetrazolium salt [29]. The glutathione peroxidase assay kit measures GPx activity indirectly by a coupled reaction with GR [30]. Also, a thiobarbituric-acid-reactive substance assay was used to measure the lung lipid peroxidation products, malondialdehyde (MDA) equivalents [31].

Western blot

Lung samples of three rats from each group were taken 3 minutes after the last administration. The samples were added with the lysis buffer on the ice for cracking for 1 hour and then centrifuged at 16,009.2× g to obtain the supernatant. The tissue protein concentration in the supernatant was determined by bicinchoninic acid (BCA) method, and 10% SDS-PAGE gel electrophoresis was used to isolate β-actin, iNOS and p38 MAPK. The proteins were transferred onto PVDF membrane for 2 hours, and the membrane was rinsed with Tris buffer saline Tween (TBST) for 5 minutes and then blocked with the blocking buffer for 1 hour. After the incubation at room temperature, the blocking buffer was discarded. The first antibodies of iNOS (1:1000), β-actin (1:1000) and p38 MAPK (1:1000) Santa Cruz Biotechnology, Inc, Calif, USA) were added onto the membrane, respectively, which was incubated at 4°C overnight and then washed with TBST five times, 5 minutes each time; the second antibody (1:2,000) were added onto the membrane, which was incubated for 2 hours and then washed five times with TBST, 5 minutes each time, and finally, ECL color solution was added onto the membrane for its development.

Histological assessment

The lung was sliced, and pieces were fixed in 10% buffered formaldehyde solution for histological study. Sections of 5 ml in thickness were prepared and then stained with hematoxylin and eosin for light microscopy analyses according to the method of Bancroft and Steven [32].

Statistical analysis

The results were expressed as mean ± SD. All the data were statistically evaluated with SPSS/18 Software. P values of < 0.05 were considered statistically significant.

III. Results

Table (1): Effect of Spirulina platensis (SP) extract on plasma lipid profile in rats treated with nicotine

Groups	Treatment Description	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-Cholesterol (mmol/L)	LDL-Cholesterol (mmol/L)
I	Normal control 3 mL of distilled water, orally	92.6 ± 3.17[a]	65.37 ± 2.97[a]	33.60 ± 2.57[a]	45.93 ± 6.50[a]
II	3% SP water extract	94.20 ± 3.80 [a]	64.80 ± 3.86[a]	32.09 ± 4.30[a]	49.15 ± 3.87[a]
III	6 % SP water extract	93.17 ± 4.27[a]	63.08 ± 4.58[a]	32.96 ± 3.27[a]	47.6 ±3.27[a]
IV	Nicotine 2.5 mg/kg b.w. in tween 80, 1%, subcutaneous injection	136.76 ± 4.11[b]	97.50 ± 3.89[b]	19.80 ± 2.43[a]	97.46 ± 4.55[b]
V	SP (3%) + Nicotine	94.00 ± 4.35 [a]	65.40(13.08 ± 3.88[a]	30.87 ± 4.39[a]	50.05 ±4.17 [a]
VI	SP (6%) + Nicotine	93.57 ± 6.10[a]	65.98 ± 4.76[a]	32.70 ± 3.80[b]	47.67 ± 4.25[a]

Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at $P \leq 0.05$.

Tables (1) show plasma lipid profile levels. Nicotine administration led to significant increase of biochemical marker levels for cholesterol, triglycerides and LDL-cholesterol while significantly decreasing HDL-cholesterol, respectively, as compared with the normal control group ($P < 0.05$), indicating acute lung injury. Treatment of animals with SP extract 3 and 6%, significantly reduced the level of cholesterol, triglycerides and LDL-cholesterol as well as significantly increased HDL- cholesterol, respectively, ($P < 0.05$), as compared with the nicotine treated group.

Table (2): Effect of Spirulina platensis (SP) extract on levels of serum tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and growth factor (TGF)-β1 in rats treated with nicotine

Groups	Treatment Description	TNF-α (pg/ml)	IL-10 (pg/mL)	TGF- β1 (pg/mL)
I	Normal control 3 mL of distilled water, orally	15.64 ± 1.65[a]	12.65 ± 1.26 [a]	63.54 ± 4.00 [a]
II	3% SP water extract	15.34 ± 1.54[a]	11.54 ± 4.21 [a]	62.10 ± 2.65 [a]
III	6% SP water extract	14.65 ± 1.90[b]	11.00 ± 5.33[a]	60.43 ± 3.64[a]
IV	Nicotine 2.5 mg/kg b.w. in tween 80, 1%, subcutaneous injection	58.70 ± 3.20 [d]	26.54 ± 2.00 [c]	197.60 ± 11.25 [d]
V	SP (3%) + Nicotine	20.54 ± 1.44[b]	14.30 ± 1.06 [b]	84.30 ± 6.50 [b]
VI	SP (6%) + Nicotine	15.80 ± 1.06[c]	11.60 ± 1.20 [b]	62.30 ± 5.40 [c]

Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at $P \leq 0.05$.

Table (2) revealed a significant elevation in plasma TNF-α and IL-6 as well as significant decrease in TGF-β1 levels ($p<0.05$) in the nicotine (2.5 mg/kg) treated group compared with the control group. The administration of SP extract 3 and 6% showed significantly decreased in TNF-α and IL-6 as well as significant increase in TGF-β1 levels relative to nicotine treated group after 30 days ($p< 0.05$).

Table (3): Effect of Spirulina platensis (SP) extract on activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and total protein kinase-1 (Akt-1) as well as malondialdehyde (MDA) level in lung rats treated with nicotine

Groups	Treatment Description	SOD (U/mg protein)	GPx (U/mg protein)	Total Akt-1 (ng/mg protein)	MDA (nmol/ mg protein)
I	Normal control 3 mL of distilled water, orally	15.4 ±1.66[a]	11.7 ±0.65[a]	3.73 ±0.84[a]	1.70 ± 0.09[a]
II	3% SP water extract	15.40 ±1.20[a]	11.80 ±0.95[a]	3.90 ±0.55[a]	1.75 ± 0.09[a]
III	6% SP water extract	16.54 ± 1.43[b]	12.65 ± 1.43[a]	4.10 ± 0.36[a]	1.69 ±0.54[a]
IV	Nicotine 2.5 mg/kg b.w. in tween 80, 1%, subcutaneous injection	8.25 ±0.94[b]	6.94 ±0.25[b]	1.90 ±0.44[c]	3.33 ± 0.14[b]
V	SP (3%) + Nicotine	16.80 ±1.15[a]	12.00 ±1.07[a]	3.50 ±0.43[a]	1.65 ± 0.06[a]
VI	SP (6%) + Nicotine	14.25 ±1.07[a]	9.00 ±0.80[a]	3.45 ±0.65[b]	1.60 ± 0.11[a]

Values are given as mean ± SD for groups of six animals each. Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at $P \leq 0.05$. SOD: one unit of activity was taken as the enzyme reaction, which gave 50%, inhibition of NBT reduction in 1min/mg protein; GPx: µg of GSH consumed/min mg protein

Table (3) show a significantly ($P < 0.05$) decreased activities of lung antioxidant enzymes SOD, GPx and total Akt-1 while significantly increasing lung MDA, were observed in the nicotine-treated rats as compared with the normal control group ($P<0.05$), indicating acute lung damage. SP extract 3 and 6% treatment significantly ($P < 0.05$) enhanced the lung enzymes activities SOD, GPx and total Akt-1 in rats and decrease MDA level, as compared to the nicotine-treated group.

Figure (1) displayed that nicotine (2.5 mg/ kg) promoted the iNOS protein expression in nicotine- treated rats compared with control group. Administration of SP extract 3% and 6% led to a statistically significant decrease of iNOS protein expression relative to nicotine treated group of rats ($p< 0.05$). Agarose gel electrophoresis images of iNOS and β-actin by RT-PCR support the present results **Figure (3)**.
Figure (2) showed that significant increase in the expression levels of p38 MAPK in group of treated rats with nicotine (2.5 mg/ kg) when compared with normal control group of rats. Also, Administration of SP extract 3% and 6% led to a statistically significant decrease of p38 MAPK protein expression relative to nicotine treated rats ($p< 0.05$). Agarose gel electrophoresis images of p38 MAPK and β-actin by RT-PCR support the present results **Figure (3)**.

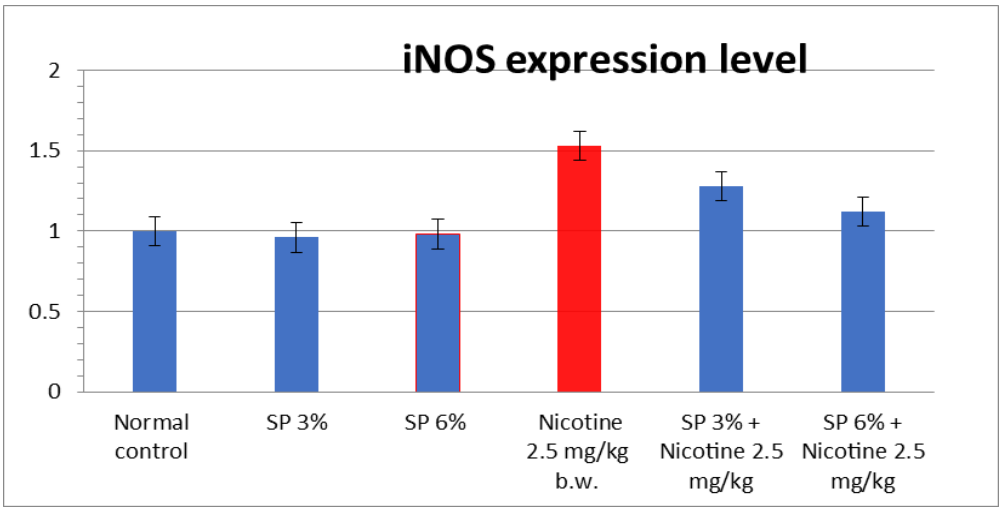

Figure 1: Effect of *Spirulina platensis* (SP) extract on levels of lung inducible nitric oxide synthase (iNOS) in rats. Representative bar diagram of three independent experiments are presented

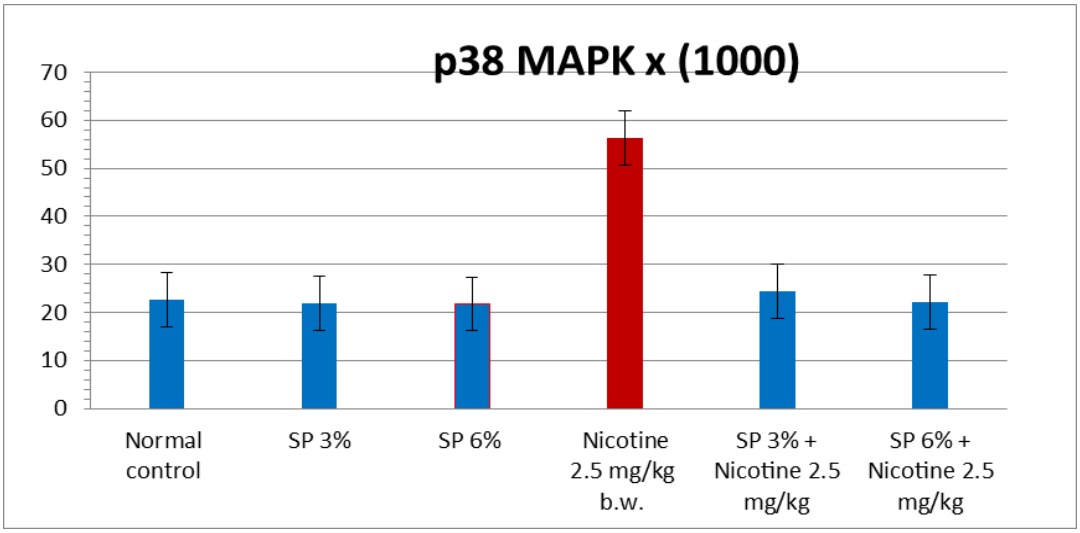

Figure 2: Effect of *Spirulina platensis* (SP) extract on lung phosphospecific p38 mitogen-activated protein kinase (p38-MAPK) in rats. Representative bar diagram of three independent experiments are presented.

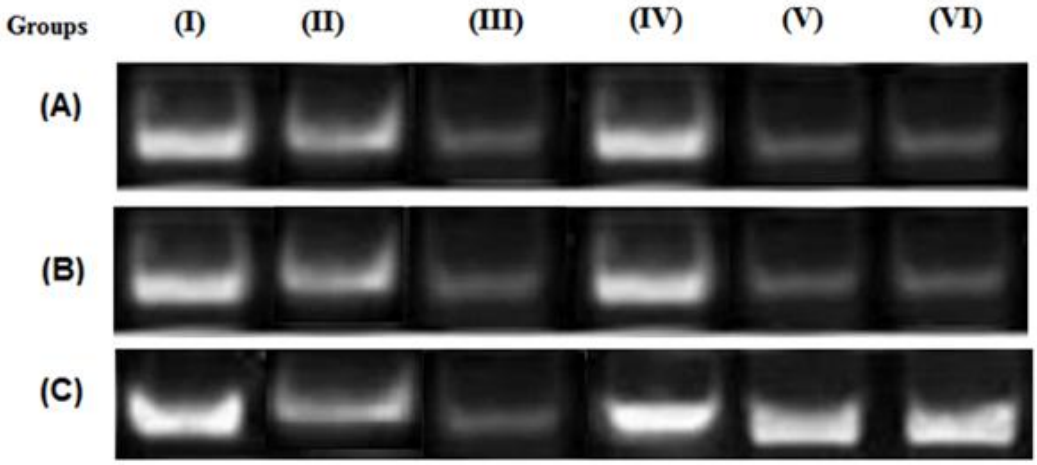

Figure 3: An agarose gel electrophoresis shows (A) PCR products of lung inducible nitric oxide synthase (iNOS), (B) p38 mitogen-activated protein kinase (p38-MAPK) and (C) β actin in different studied groups

Histopathology examination

Figure (4) show histopathological examination of lung sections of the normal groups (I) as well as SP extract 3% and 6% treated group (II and III) demonstrated normal morphological features of lung parenchyma with apparent intact respiratory airways epithelium as well as alveolar walls with intact vasculatures.

On the other hand, in the lung of nicotine-treated control group (IV), histological examination showed marked diffuse hemorrhagic pneumonia with extravasation of blood into alveolar lumen and intra-bronchiolar lumen accompanied with sever thickening of interalveiolar walls and peribronchiolar tissue with many inflammatory cells infiltrates.

Histopathological examination also showed good recovery of nicotine-induced lung toxicity (V) by SP extract 3% as compared with the nicotine-treated group.

Group (VI) all samples of nicotine treated rats recovery by treatment with SP extract 6% and showed almost intact morphological features of pulmonary tissue with minimal records of inflammatory cells infiltrates.

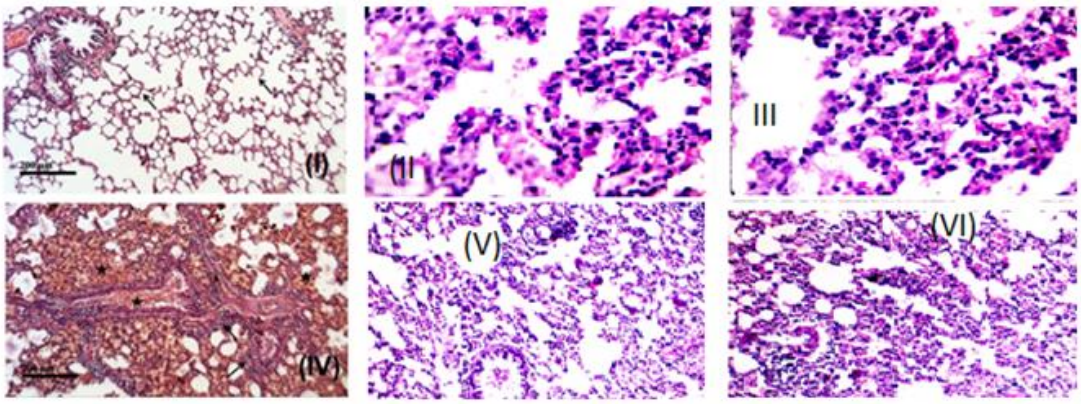

Figure (4): Histopathological examination of lung sections of the normal groups (I) as well as SP extract 3 and 6% treated group (II and III) demonstrated normal morphological features of lung parenchyma with apparent intact respiratory airways epithelium as well as alveolar walls (arrows) with intact vasculatures. On the other hand, in the lung of nicotine-treated control group (IV), histological examination showed marked diffuse hemorrhagic pneumonia with extravasation of blood into alveolar lumen and intrabronchiolar lumen (stars) accompanied with sever thickening of interalveiolar walls and peribronchiolar tissue with many inflammatory cells infiltrates (arrow). Histopathological examination also showed good recovery of nicotine-induced lung toxicity (V) by SP extract 3% as compared with the nicotine-treated group.Group (VI) all samples of nicotine treated rats recovery by treatment with SP extract 6% and showed almost intact morphological features of pulmonary tissue with minimal records of inflammatory cells infiltrates.

IV. Discussion

Spirulina platensis (SP) has the ability to modulate immune functions and exhibits anti-inflammatory properties by inhibiting the release of histamine from mast cells. Many studies suggested that these algae may also improve several diseased conditions and may even have antiviral effect [17]. It has been suggested that the ability of SP to inhibit carcinogenesis is due to its antioxidant properties that protect tissue from cell damage [18].

Smoking ranks among the top causes of cardiovascular disease, including coronary heart disease, ischemic stroke, peripheral artery disease and abdominal aortic aneurysm [33]. It is also associated with an increased risk of certain types of cancer and is a major cause of chronic obstructive pulmonary disease [34].

In the present study, the levels of total cholesterol and triglycerides and low-density lipoprotein (LDL) by rats during experimental period were significantly increased in nicotine-treated rats when compared with normal control rats. On the other hand, nicotine lowers plasma levels of high-density lipoprotein (HDL), a powerful protective factor against the development of atherosclerosis.

Also, oral administration of SP at 3% and 6% respectively, showed significant protection against nicotine induced increase in plasma cholesterol, triglycerides and LDL. The deposited cholesterol esters in the tissue need hydrolysis to release free cholesterol. One of the hydrolysis factors is HDL, since HDL-cholesterol level was found to be decreased in atherogenic diet fed rats [35], the insufficient HDL level may lead to free cholesterol in plasma, enhancing the pathogenesis. The results of the present work showed that Spirulina platensis extract enhanced HDL in treated rats. The most obvious effect of resveratrol on lipid profile was its action on in vivo LDL. SP reduced the LDL in nicotine treated rats. LDL promotes atherosclerosis both by providing lipids signals that initially activate macrophages, and by stimulating foam cell formation [35].

According to Nagaoka et al. [36], phycocyanin caused hypocholesterolemic activity in rats. They hypothesized that phycocyanin binds to bile acids in the jejunum, this binding affects the micellar solubility of cholesterol and then suppresses cholesterol absorption. Seo et al. [37] reported that - carotene reduced the elevation of cholesterol and triglycerides of diabetic rats. Both sulfated polysaccha-rides and linolenic acid showed hypolipidemic effect [38, 39]. Moreover, Kim et al. [40] found that feeding of rats with linolenic acid rich oil lowers plasma triacylglycerol and in hibits hepatic fatty acid synthesis which may result in a hypolipidemic effect.

The present results showed that SP could inhibit serum TNF-α, IL-6 and TGF-β1 levels in the nicotine-treated group. Free radicals are involved in the regulation of cell proliferation and death, as well as gene expression such as TNF-α, IL-6, TGF-β1 and MDA [41]. Evidence indicates that free radicals, oxidative stress, and lipid peroxidation are present in organs damage [42]. It has been shown that in chronic lung toxicity, the increased lung concentration of as TNF-α, IL-6, TGF-β1 and MDA and decrease activity of SOD, GPx and Akt-1 induces mitochondrial toxicity and free-radical generation [43]. TNF-α, TGF-β1, and interleukin-6 are the most extensively studied mitogenic and fibrogenic factors.

In addition to the induction of cytokine expression, NF-κB also transcribes inhibitor of kappa B (IκB), as a part of an autoregulatory loop, which can bind p65 in the nucleus for exiting p65 back into the cytoplasm [44].

It is unknown whether adipocytes possess this autoregulatory loop for the resolution of inflammatory responses. It is tempting to speculate that adipocytes may not have a self-resolving capacity, leading to extended residency of NF-κB in the nucleus for ultimate repression of adipocyte differentiation. This aspect warrants further investigation.

In an earlier experiment to determine the radical scavenging activity of C-phycocyanin isolate of S. platensis, an intraperitoneally administered C-phycocyanin was found to reduce the peroxide values of CCl4-induced lipid peroxidation in rat liver microsomes [45]. Following a study conducted on 60 patients presenting with chronic diffuse disorders in the liver and on 70 experimental animals, Gorban et al. [46] have found that Spirulina administration prevented the transformation of chronic hepatitis into hepatic cirrhosis. Recently, Paniagua Castro et al. [47] have demonstrated the protective efficacy of Arthrospira against cadmium-induced teratogenicity in mice.

Supporting the present study results, the study reported that in lung toxicity by nicotine, iNOS begins to express and generate a large number of NO, and endogenous NO is massively released into regional damaged lung tissue, which directly react or interact with other factors, indirectly involved in scar formation and evolution process by adjusting firoblasts, endothelial cells and other functions [48].

There are more major findings in the resveratrol study. First, the present results shown that TNF-α-induced increased monocyte adhesiveness to Human Coronary Artery Endothelial Cells (HCAECs) is NF-κB dependent, and it can be inhibited by SP [46]. IL-10 and iNOS protein expression also elicited endothelial activation, and this effect also could be attenuated by SP. It is significant that SP also attenuated H_2O_2-induced monocyte adhesion to HCAECs in a similar concentration range [49]. The second important finding is that TNF-α -induced NF-κB activation in HCAECs is inhibited by treatment with SP [47]. The present study was confirmed with other studies [45-47] suggested that resveratrol was effective against iNOS protein expression, IL-10 and TGF-β1-induced NF-κB activation in intact blood vessels.

In lung cells, TNF-α, IL-6, TGF-β1 can induce P38MAPK activation and increase its activity, thereby inducing cardiac endothelial cell death and stimulating neutrophil function, leading to the increase of TNF-α, IL-6, TGF-β1 -α and the accumulation of neutrophils in lung tissue and damage it's cells [46]. Baicalin significantly down-regulated P38MAPK protein expression in rat with nicotine induced lung toxicity group. Piero et al. concluded that treatment with resveratrol suppresses migration, invasion and metastasis through p38MAPK signaling pathway in human cardiac tissue [50].

Indeed, there was remarkable reduction in fibrosis extent and a decrease of stellate infiltration in rats treated with SP groups compared to the nicotine treated group. Histological studies confirmed the lung protective effect of SP. Since the proliferation of lung is an early event in toxicity-related changes, the attenuation of lung injury and fibrosis in rats by SP might be associated with alleviation of inflammatory reaction. Prophylactic effect of SP against nicotine-induced lung toxicity has not been reported earlier to our knowledge, and this study is perhaps the first observation of its kind.

V. Conclusion

The present study showed that Spirulina platensis extract has powerful lung protective activity against nicotine-induced lung toxicity; via normalize the levels of oxidative stress biomarkers and gene expression of inflammatory mediators.

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