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Study the Effect of Total Carotenoids from *Sporidiobolus pararoseus* on Acute Lung Inflammation in Mice Exposed to Cigarette Smoke

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Abstract Cigarette smoke (CS) is a major risk for chronic obstructive pulmonary disease (COPD). Pathogenesis hallmarks in the lungs of COPD patients always contribute to causing inflammation and oxidative stress. Carotenoids, extracted from *Sporidiobolus pararoseus*, have antioxidant and antitumor effect. These carotenoids have a similar structure to lycopene, which is demonstrated effective in the treatment of lung inflammation. This presented work aims to investigate the anti-inflammatory and antioxidant functions of total carotenoids (TC), extracted from *Sporidiobolus pararoseus*, on mice under a short-term exposure of cigarette smoke. Forty-eight C57BL/6 male mice were divided into 6 groups (n=8): first group was control group exposed to ambient air, second group was CS group exposed to CS, third group was CS+LY18 group exposed to CS and treated with lycopene 18 mg/kg (CS+LY18), other groups (CS+TC18, CS+TC12, CS+TC9) exposed to CS and treated with (TC) at different doses (18, 12, 9 mg/kg) respectively. Treatment with TC especially at high dosage 18 mg/kg led to decrease the levels of MDA, GSH, activities of CAT, SOD in lung samples, as well as TNF-α and IL-6 levels in lungs and bronchoalveolar lavage fluid. In addition, it attenuated the morphological changes in the lungs. The high dose of TC showed a stronger effect than lycopene. These results demonstrated that TC from *Sporidiobolus pararoseus* have effective functions for acute lung inflammation induced by CS, which suggested a positive intervention for the treatment of COPD.

Keywords: *Sporidiobolus pararoseus, total carotenoids, COPD, cigarette smoke, inflammation, oxidative stress*

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is a global health problem with high morbidity and mortality, anticipated to be the third leading cause of death in the world by 2020 [1]. COPD is caused by several risk factors such as indoor/outdoor air pollution and cigarette smoke (CS), of which CS is considered the most powerful and major cause of this disease [2]. The CS-induced COPD is mainly characterized by slow and irreversible deterioration of lung function due to chronic inflammation [3]. There are two reasons for increasing the number of alveolar oxidants that are responsible for the inflammation in the lung. The first one is that CS stimulates increasing the number of inflammatory cells in alveoli. The second reason is that CS contains an expressive number of free radicals [4]. The presence of inflammatory cells, cytokines (tumor necrosis factor-α TNF-α, interleukin-6 IL-6) and redox markers in smoker's lung leads to oxidative stress and inflammation [5]. Antioxidants have an important role in reducing the oxidative damage and the number of inflammatory cells [6], so that could attenuate the injury of CS-induced lung inflammation [7].

Carotenoids are a family of more than 600 compounds of the most common pigments in nature [8]. Several studies have demonstrated the effect of carotenoids as antioxidants that protect the body from acute and chronic diseases [9]. In addition to their primary role as an antioxidant, carotenoids also have individual functions such as prevention of scaly skin, retarded tooth, eye diseases and cardiovascular diseases [10]. This significance led to an increased commercial interest in the search for

alternative natural sources. Microbial production can overcome seasonal problems and geographic variability compared with the extraction from vegetables, and by this process, synthesis of carotenoids can be done from low–coast source like carbohydrate, which gives additional importance to microbial production as a suitable option for industrial applications [11]. *Sporidiobolus pararoseus* (SP) belongs to the class of Urediniomycetes which can biosynthesis abundant intracellular carotene (β-carotene, γ-carotene, torulene and torularhodin). The chemical structures of carotenoids in SP (as we see in supplementary information, especially torularhodin and torulene) are similar to lycopene [12]. Some studies demonstrated the role of lycopene in the prevention of acute lung inflammation as an antioxidant and anti-inflammatory [13,14]. Our previous reports showed the potential function of some carotenoids from SP as antioxidant and anticancer [15,16,17], but there is no research about TC from SP towards CS-induced COPD.

The aim of this study is to investigate the effect of TC isolated and purified from SP, as an anti-inflammatory and antioxidant on acute pneumonia induced by mice exposed to short-term cigarette smoking.

2. Materials and Methods

2.1. Materials

Sporidiobolus pararoseus (JD-2 CCTCC M 2010326) was obtained and characterized in our laboratory. TNF- α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Sbjbio company (Nanjing, China). Other commercial kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).The lycopene standard (98% purity) was obtained from Sigma Company (Shanghai, China). HPLC reagents and other chemical reagents were purchased commercially with high quality.

2.2. Preparation of Total Carotenoids

The cultivation of SP was prepared according to Han Mei's method [18]. In the extraction environment, we must avoid high temperature, light, oxygen and pro-oxidant metal like copper and iron to prevent oxidation of carotenoids [19]. Preparation of crude pigments was according to Qiuyu Shi's method [12]. Briefly, at first, the cells were harvested by centrifugation (12000 rpm, 20 min, 4°C), then washed with distilled water, resuspended in the same volume of Dimethyl sulfoxide (DMSO) in a shaking bath to increase the extraction qualification. Then, the colored solution was centrifuged (8000 rpm, 10 min, 4°C) to separate and collect it. This step was repeated several times. Then, acetone was added and the mixture was centrifuged (8000 rpm, 10 min, 4°C), and this treatment was repeated until the color decay. The colored layer was collected and water was added to remove acetone. Finally, the crude pigments were concentrated by evaporation, flushed with the nitrogen atmosphere and stored at -20°C. Crude pigments were applied to a silica gel column (100 – 200 mesh) for purification and eluted using acetone and hexane. Thin-layer chromatography (TLC) was used to identify the similar fractions. To increase purity, the pigments were separated 2-3 columns. High Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) was used to determine the carotenoids [12]. HPLC conditions were as follows: mobile phase solvent A: acetonitrile/water/formic acid (86: 10: 4 v/v/v) and solvent B: ethyl acetate/formic acid (96: 4 v/v), gradient elution: during 0-25 minutes solvent A to 100% and solvent B to 100%, then changed to solvent A 100%, for last 5 minutes [20], with flow rate: 1.0 mL/min, injection volume: 10 μL and column temperature: 25°C. Carotenoids separated on Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μL). The wavelengths ranging of the UV-visible spectrum was 200-600 nm. The carotenoids were defined by using β-carotene as standard due to unavailability of commercial standards of others. The purity of TC was about 85%.

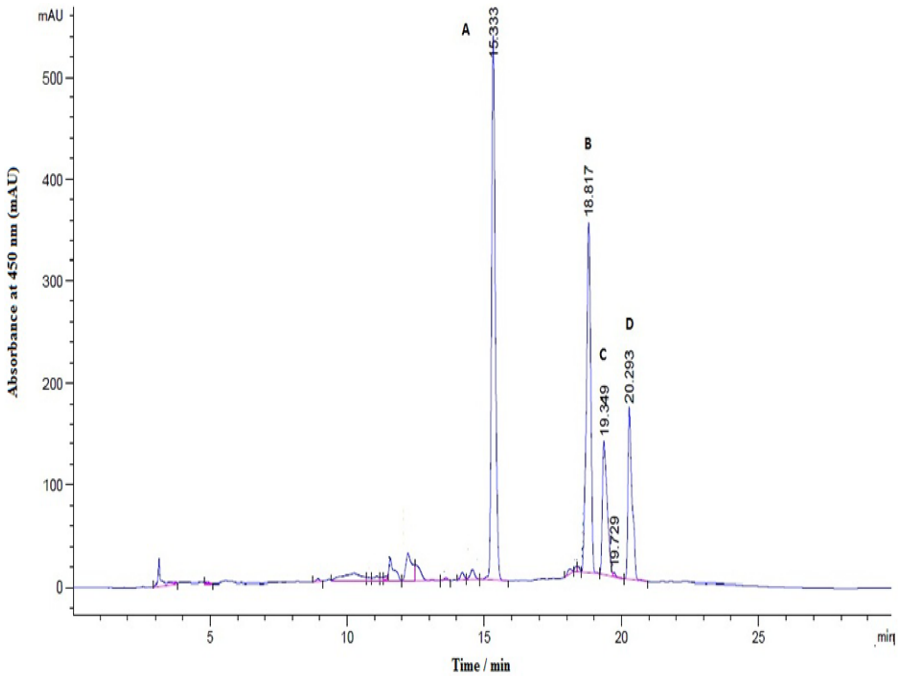

Figure 1. HPLC for TC from *SP*: A: Torularhodin, B: Torulene, C: γ-carotene, D: β–carotene.

2.3. Animals

Forty-eight (48) C57BL/6 male mice 8 weeks old (20-22 g) were purchased from Shrek company (Shanghai, China). The animals had free access to water and food of commercial standard chow and housed under set temperature (23±2°C) and humidity (60±5%) with a 12-h light/dark cycle. After one week of acclimatization, the mice were divided into 6 groups, each group contained8 mice, 4mice/cage: control group, CS group, CS+Lycopene 18 mg/kg (CS+LY18), CS+TC at high dose 18 mg/kg (CS+TC18), CS+TC at middle dose 12 mg/kg (CS+TC12) and CS+TC at low dose 9 mg/kg (CS+TC9). Both the control group and CS group treated with vehicle.TC and lycopene were administered intragastrically by gavage one time every day after dissolve them in corn oil. All the principles of laboratory animal care were followed in compliance with the Chinese Experimental Animals Administration Legislation and were approved by the Science and Technology Department of Jiangsu Province.

2.4. Cigarette Smoke Exposure

To study the effect of TC in CS-exposed mice, (48) C57BL/6 male mice were exposed to 6 commercial full-flavored Marlboro cigarettes (10 mg tar, 0.9 mg nicotine and 10 mg monoxide) per day for 5 continuous days in a smoking chamber [21,22]. Briefly, mice were placed group by group in the inhalation chamber (40 cm long, 30 cm wide and 25 cm high), inside an exhaust hood. A cigarette was coupled to a plastic 60 mL syringe so that puffs could be drawn in and subsequently expelled into the exposure chamber. One liter of smoke from each cigarette was aspirated with this syringe (20 puffs of 50 mL each), and each puff was immediately injected into the smoking chamber. CS groups were maintained in this condition of smoke-filled air (~3%) for 6 minutes. The exhaust fan was turned off. Then the cover of the chamber was removed and the exhaust fan was turned on to evacuate the smoke. This cigarette exposure was repeated two times (2cigarette×6 min), so every period of the day had a total of 12 min CS exposure. This procedure was repeated 3 times per day (2 cigarettes in the morning – 2 cigarettes at noon –2 cigarettes in the afternoon) which resulted in 36 minutes of CS exposure per day. Each cigarette produces 300 mg/m^3 of total particulate matter in the chamber, (measured by weighing material collected on Pallflex filters). The concentration of Carboxyhemoglobin (COHb), previously monitored in mice in the same experimental protocol, was not toxic [23]. Control group was exposed to ambient air under the same conditions.

2.5. Collection of Bronchoalveolar Lavage Fluid (BALF)

One day after the final ambient air or CS exposure, all mice were sacrificed by cervical dislocation. BALF was performed in the left lung of all animals in each group. Briefly, a cannula was inserted into the trachea and the air spaces were washed with a buffered saline solution (500 μL) three times, and the flow-through (final volume 1-1.2 mL) was maintained on ice. Total cells in BALF were determined using the Trypan blue exclusion method.

2.6. Tissues Preparation

After collection BALF, the lungs were immediately removed and homogenized on ice with 10% (w/v) PBS, and the homogenates were centrifuged at 4000 for 20 min. Supernatants were stored at − 20°C for analysis of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione (GSH). Total protein was determined by using BCA kits.

2.7. Histological Analysis

The right ventricle was perfused with saline to remove blood from the lungs. The right lungs were collected and fixed in 4% paraformaldehyde fixing solution for 48 hours. Sections (5 μm) were stained with hematoxylin and eosin. A light microscope was used to study histological changes.

2.8. CAT, SOD and GSH

The activities of antioxidant enzymes (CAT and SOD) and levels of GSH in lung homogenates were determined using the commercial kits according to the manufacturer's instruction, measured at 405 nm, 450 nm and 405 nm respectively.

2.9. Malondialdehyde Assay

As an index of oxidative damage induced by lipid peroxidation, we used the thiobarbituric acid reactive substances (TBARS) method to analyze the production of malondialdehyde during an acid-heating reaction as previously described by Draper et al. [24] Samples from lung homogenates were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid; then samples were heated in a boiling water bath for 30 min. TBARS levels were determined by absorbance at 532 nm and expressed as malondialdehyde equivalents.

2.10. Measurement of Pro-inflammatory Cytokines

After cutting samples, PBS (PH7.2-7.4) was added, then samples were homogenized by hand or grinders, centrifuged 20 min at the speed of 2000-3000 r.p.m, and supernatant was removed. The levels of IL-6 and TNF-α in lung homogenates and BALF were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol and then the absorbance was read at450 nm.

2.11. Statistical Analysis

Values for all measurements are expressed as means±Standard deviation. Statistical significance was determined using one-way ANOVA followed by the Duncan post-hoc test. The p value <0.05 was considered significant.

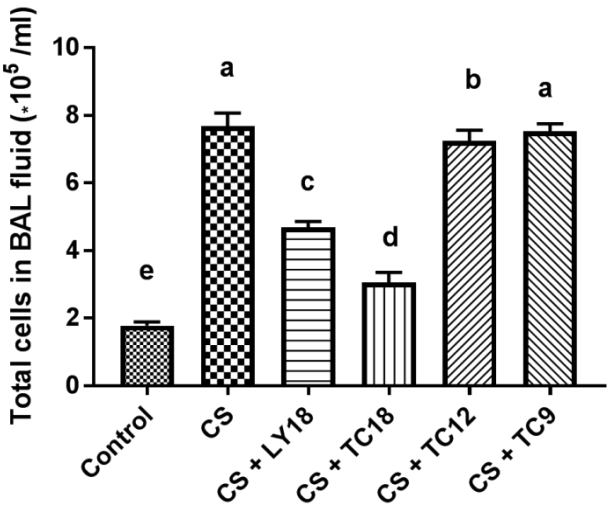

Figure 2. Effect of TC on the total cells in BALF. The control group (mice exposed to room air and treated daily with corn oil). CS group (mice exposed to 6 cigarettes per day for 5 continuous days and treated with corn oil). CS+LY18 (mice exposed to 6 cigarettes per day for 5 continuous days and treated with lycopene 18 mg/kg). CS+TC18, CS+TC12, CS+TC9 (mice exposed to 6 cigarettes per day for 5 continuous days and treated with TC 18, 12, 9 mg/kg respectively). We performed one-way ANOVA followed by the Duncan posttest ($p<0.05$, $n=8$) to compare between groups. Different letters appear statistically significant differences between groups, $p<0.05$. Data were expressed as Means ±SDs

3. Results

3.1. Total Cells Number in BALF

Exposed mice to CS for five continuous days showed an increase in the total cells number in BALF compared to the control group (Figure 2). Administration with TC significantly reduced the total number of cells and was close to the control group. The best effect was obtained at the highest dose and more strongly than lycopene. This data suggest that TC18 mg/kg can attenuate inflammatory cell infiltration in acute lung inflammation induced by CS.

3.2. Histological Analysis of Lung Tissues

The histological changes of the lung were studied by H&E staining and using the light microscope to check the results. In the control group, we can find thin alveolar septa and normal sized-airspace (Figure 3A), whether we can see in CS group increasing of inflammatory cells and thickening the bronchial wall (Figure 3B). On another hand, treatment with TC at the highest dosage decreased the inflammatory cells and the signs of lung inflammation superior to lycopene (Figure 3C-D). In contrast, CS+TC12 and CS+TC9 were similar to the CS group with fewer leukocytes (Figure 3E-F). The lung morphologies in the CS+TC18 mg/kg are similar to that in the control group.

3.3. Effect of TC on SOD and CAT Activity

Our data showed that exposure to CS led to an imbalance between oxidants/antioxidant. As depicted in (Figure. 4), CAT activity in lung homogenates increased after CS exposure in comparison to the control group. The treatment with TC at all doses decreased the level of catalase. Notably, the highest dosage exerted a stronger effect better than lycopene. SOD activity was higher in the CS group (Figure 4). In contrast, the SOD levels in a group treated with TC18 was similar to the control group and no significant difference between CS+LY group and CS+TC18 group, whereas the treatment with TC at low and middle doses didn't change the values of SOD compared to CS group. The experimental data suggested that TC from SP could relieve oxidative stress in acute lung inflammation.

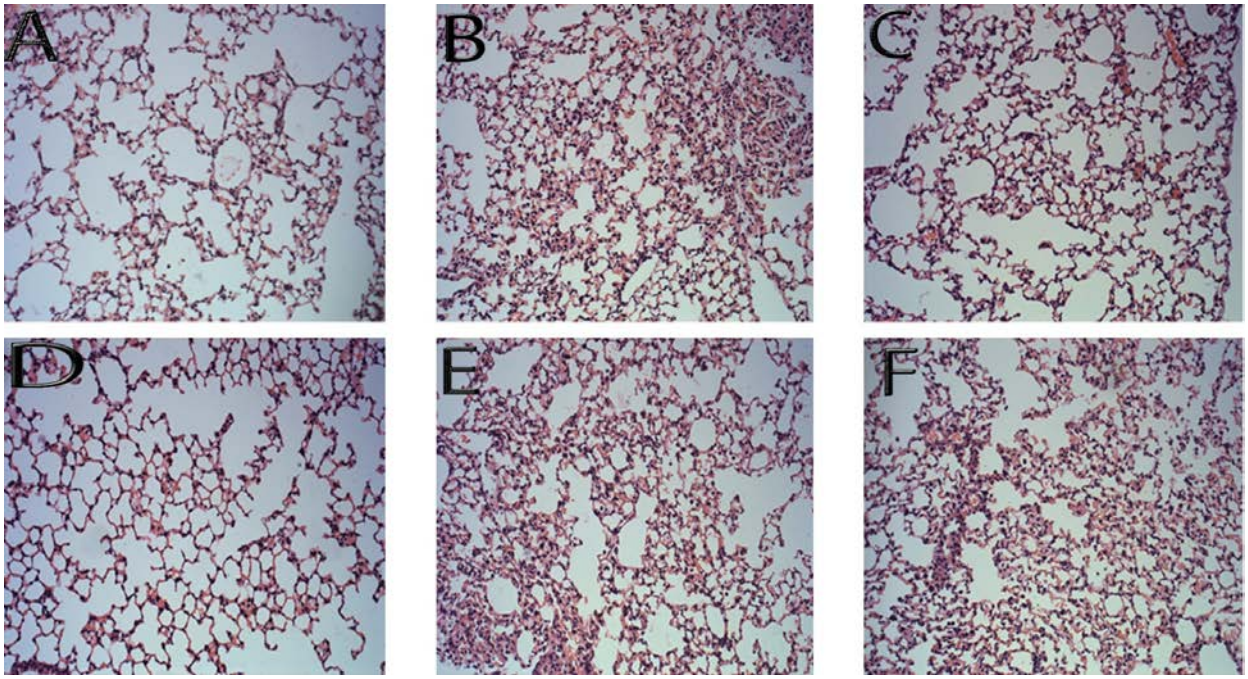

Figure 3. Photomicrographs of lung tissues after staining with hematoxylin and eosin. A: control group without smoking exposure, B: CS group: 6 cigarettes/day for five continuous days, C: mice exposed to CS and treated with Lycopene 18 mg/kg, D: mice exposed to CS and treated with TC 18 mg/kg, E: mice exposed to CS and treated with TC 12 mg/kg, F: mice exposed to CS and treated with TC 9 mg/kg

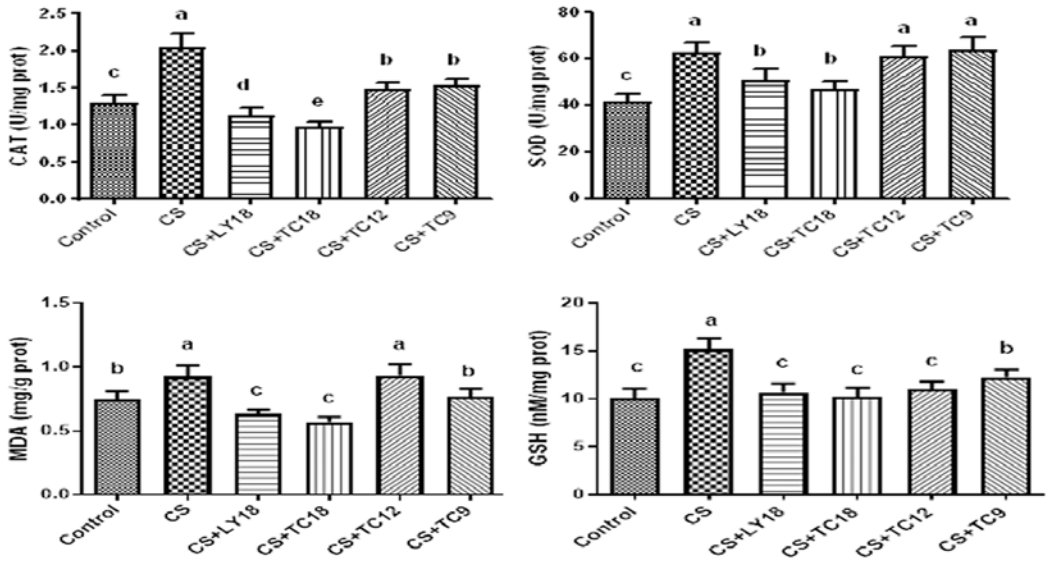

Figure 4. Assessment of the TC effect on oxidative stress, by measuring the MDA, GSH levels and the activity of SOD, CAT in lung homogenates from all mice. To compare between groups, we performed one-way ANOVA followed by the Duncan posttest ($p<0.05$, n=8). Different letters appear statistically significant differences between groups. Data were expressed as Means ± SDs

3.4. Effect of TC on MDA

Exposed mice to CS increased the level of MDA as compared with those in the control group (Figure 4). However, reduction in MDA production was noticed in the CS+LY and CS+TC18 in comparison to the mice in the CS group, but no significant difference was seen between both of them. The results demonstrated that TC at a high dose suppressed the rising of MDA level, and further weakened the peroxidation harm instigated by tobacco smoke.

3.5. Effect of TC on GSH Level

GSH level was increased in the model group compared to control group (Figure 4). The treatment by TC completely restored GSH content compared to mice in the control group. The effect of TC was similar to the lycopene. Our data showed that antioxidant prosperities of TC extracted from SP.

3.6. Effect of TC on Pro-inflammatory Cytokines

To evaluate the anti-inflammatory effects of TC from SP, we detected the production of TNF-α and IL-6 in BALF and lung samples by ELISA. As shown in (Figure 5), model group exhibited a marked increase in TNF-α and IL-6 in relation to those in the control group. The results showed that the treatment of TC seemed to reduce the generation of these pro-inflammatory factors in BALF and pulmonary tissue. As expected, we observed the best effect in animals administered 18mg/kg TC. Our finding indicated that TC exhibited anti-inflammatory properties in acute lung inflammation.

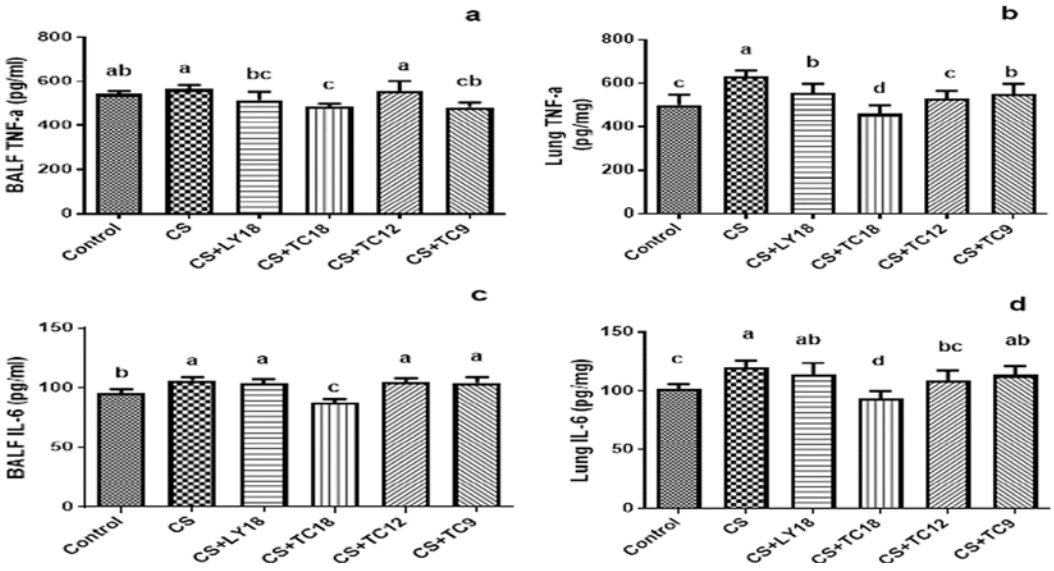

Figure 5. Evaluation the levels of pro-inflammatory cytokines measured by ELISA in all experiment groups: a- TNF-α levels in BALF, b-TNF-α levels in lung homogenates, c- IL-6 levels in BALF, d- IL-6 levels in lung homogenates. To compare between groups, we performed one-way ANOVA followed by the Duncan posttest ($p<0.05$, n=8). Different letters appear statistically significant differences between groups, $p<0.05$. Data were expressed as Means±SDs

All measurements of the experiment and the statistical difference between groups have been clarified in Table 1.

Table 1. Different letters in each testing parameter represent statistical significance among groups. Means ± standard deviation (n = 8), (p <0.05)

Parameter	Control	CS	CS+Lycopene	CS+TC18	CS+TC12	CS+TC9
TNF-α BALF	542.41±12.4^{ab}	563.91±18.2^{a}	514.18±38.92^{bc}	486.61±13.24^{c}	542.41±7.39^{a}	482.75±47.47^{bc}
IL-6 BALF	95.67±3.2^{b}	106.07±2.77^{a}	103.55 ± 3.56^{a}	87.48±3.02^{c}	104.79±3.13^{a}	104.02± .67^{a}
TNF-α LUNG	500.79±47.49^{c}	632.95±27.56^{a}	555.29 ± 40.9^{b}	460.04±40.11^{d}	530.83±34.48^{bc}	551.44±45.04^{b}
IL-6 LUNG	101.82±4.01^{c}	119.97±6.15^{a}	113.73 ± 9.91^{ab}	93.84 ± 6.14^{d}	108.56±8.69^{bc}	113.33±7.76^{ab}
CAT	1.3 ± 0.09^{c}	2.04±0.1^{a}	1.12 ± 0.11^{d}	0.9692 ± 0.07^{e}	1.49±0.08^{b}	1.53 ± 0.09^{b}
SOD	41.62 ± 3.44^{c}	62.83± 4.36^{a}	51.00 ± 4.65^{b}	47.32 ± 2.86^{b}	61.02±4.30^{a}	64.04 ± 5.23^{a}
MDA	0.75 ± 0.06^{b}	0.93 ± 0.09^{a}	0.63 ± 0.03^{c}	0.57 ± 0.04^{c}	0.93±0.09^{a}	0.771 ± 0.06^{b}
GSH	10.08 ± 0.99^{c}	15.19 ± 1.15^{a}	10.67 ± 0.92^{c}	10.22 ± 0.92^{c}	11.00 ± 0.78^{c}	12.25 ± 0.81^{b}

4. Discussion

A previous study has demonstrated the role of lycopene in the prevention and treatment of lung inflammation caused by smoking [13]. Taking into considerations, our pigments almost have a similar chemical structure to lycopene. Many studies have shown these carotenoids have antioxidant and anticancer effects [16,17]. To our knowledge, this is the first report that TC extracted from SP displays antioxidant and anti-inflammatory effects in acute lung inflammation induced by CS in mice. Previous studies have shown that short-term CS exposure model is an appropriate study regarding oxidative stress and inflammatory markers. CS has an essential role in the development of COPD [25]. The highest dose of TC has given us the best effect and sometimes superior to lycopene as an antioxidant and anti-inflammation. Oxidative stress supports high levels of enzymatic antioxidant like SOD and CAT activity [22]. The oxidative stress in the body causes an increase in the activity of antioxidant enzymes to protect the tissues and organisms, and this is what we got in our results, where SOD and CAT activities increased in mice exposed to smoking. This corroborates our findings because we observed a reduction of these levels in the treatment of TC but not to the same degree in the different dosages. The reduction of CAT, SOD activities and restore GSH levels confirms the role of TC as an antioxidant in acute lung inflammation, where TC treatment caused reduced oxidative damage. We noticed from our results that CAT activities were reduced at all treated groups, but SOD activities were reduced at high dosage treated group. Reducing CAT activity is more important than reducing SOD activity, due to superoxide anion is easier to scavenge than hydrogen peroxide which may cross the cell membrane and damage it [26]. From this viewpoint, we can ascertain the therapeutic effects of TC as an antioxidant. GSH is the most available antioxidant within cells and is important for protecting cells from oxidative stress. We observed an overproduction of GSH after CS-exposed [27,28]. This increase in GSH levels caused by smoking is called the GSH adaptive response [27]. The recovery of GSH levels was obtained after treatment with TC 18, 12 mg/kg and the lowest effect was at dose 9 mg/kg. The decrease in GSH levels reflects the role of these pigments in reducing the lung damage caused by smoking. MDA levels have been used as an appropriate indicator of lipid peroxidation relative to oxidative damage from smokers [29]. We found a remarkable increase of MDA levels in the mice exposed to CS. This oxidative mediator can lead to injuring the lung. However, the treatment with TC 18 mg/kg significantly inhibited high level of MDA in mice exposed to CS; pointing to TC can attenuate CS-induced oxidative stress in lung tissues. Many studies have demonstrated that antioxidant have a therapeutic effect on COPD [30]. So we can say according to our results that antioxidant prosperities of these carotenoids can prevent and reduce the effect of COPD. Acute lung inflammation also characterized by increasing inflammatory cells which lead to releasing inflammatory mediators like TNF-α and IL-6 which are considered modulators of inflammation [31]. We observed an increase in the number of total cells after exposure to CS and decrease in this number after treatment with TC 18mg/kg. TNF-α is one of the most important characteristics of COPD and it may also be an indication of the severity of the disease [32]. IL-6 is responsible for the onset and spread of inflammation [33]. We noticed in the CS group increasing in the levels of pro-inflammatory cytokines in lung and BALF compared to control group, whether TC induced decrease in the concentration of TNF-α and IL-6 compared to the model group. Thus it can be said that the TC is able to support the decreasing of inflammatory cytokines and attenuate the inflammatory response. In conclusion, our present data confirm for the first time that TC extracted from SP are a potent antioxidant and anti-inflammatory agent *in vivo*. This ability back to decrease CAT and SOD activities, MDA levels, IL-6 and TNF-α levels, restore GSH levels, in addition, reduce the morphological changes in lung tissues. Indeed, we achieved the best effect at 18 mg/kg and it was also better than lycopene at the same dosage. As a final point, total carotenoids extracted from SP have a therapeutic effect of acute pneumonia in a dose-dependent manner. Thus, in the future, a study should be conducted on exposure to long-term cigarette smoke, which causes COPD to test the effectiveness of these carotenoids in the treatment of inflammation and oxidative stress. Furthermore, we can detect which kind of carotenoids has the best effect on these diseases. More treatment strategies are urgently needed to control this disease.

Abbreviations

COPD: Chronic obstructive pulmonary disease
CS: Cigarette smoke
SP: Sporidiobolus pararoseus
TC: Total carotenoids
LY: Lycopene
HPLC-DAD: High Performance Liquid Chromatography with Diode-Array Detection.
TLC: Thin-layer chromatography
BALF: Bronchoalveolar lavage fluid
SOD: Superoxide dismutase
CAT: Catalase
MDA: Malondialdehyde
GSH: Glutathione
IL-6: Interleukin-6
TNF-α: Tumor necrosis factor-α

Conflicts of Interests

There are no conflicts of interests between authors.

Acknowledgements

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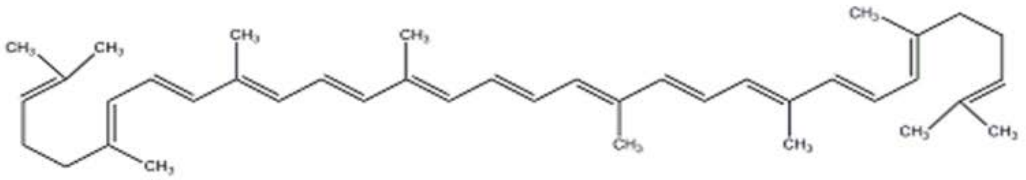
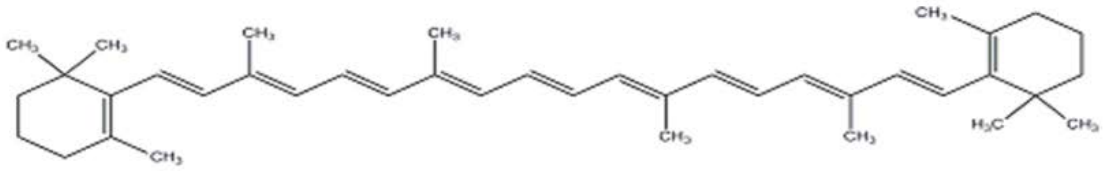
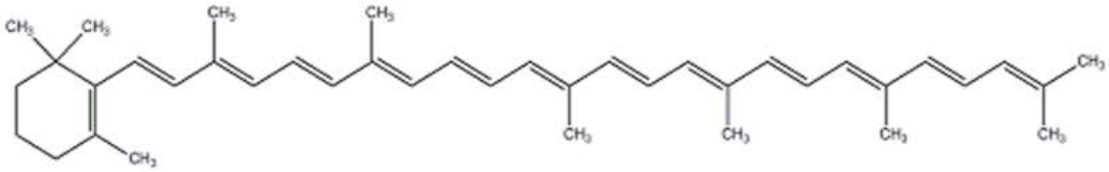
Supplementary Information



Lycopene C40H56



β-Carotene C40H56



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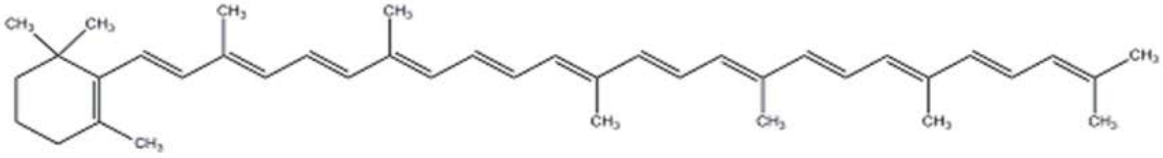
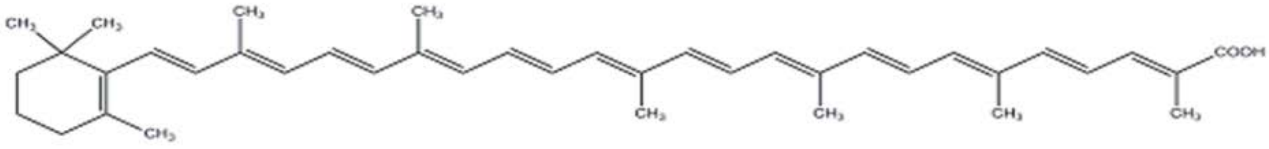


Torulene C40H54



Torularhodin C40H52O2

Chemical structures of the main carotenoids in *Sporidiobolus pararoseus*