Full Length Research Paper

Anti-oxidative and anti-inflammatory effects of *Trigonella foenum-graecum* Linnaeus, 1753 (Fenugreek) seed extract in experimental pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF) is the most common idiopathic interstitial pneumonia. A large part of research focusing on the pathogenesis of IPF suggested that oxidative stress has been implicated in the pathogenesis of bleomycin induced lung fibrosis. We therefore examined whether fenugreek *Trigonella foenum-graecum* Linnaeus, 1753 and its phenolylic extract inhibits bleomycin induced lung fibrosis in rats. Forty male Wistar rats were given a single dose of bleomycin (4 mg/kg, intratracheally). After 2 weeks of treatment, both fenugreek seed polyphenol extract (FSPE) and fenugreek powder supplementation (FPS) significantly reduced MDA (0.280±0.053 and 0.205±0.031 nmol/mg protein respectively) and increased TAS (0.888±0.086 and 0.695±0.086 mmol/l) in comparison to control groups (0.434±0.043 and 0.345±0.043 and 0.561±0.050 mmol/l for TAS). The restoration of oxidant/antioxidant balance was seen concretely through the diminution of inflammation in treated groups (3.29±0.49 and 4.29±0.76) in contrast to untreated groups (4.70±0.48 and 5.00±0.00). TGFβ was increased only in inflammatory infiltrate of parenchyma lung. In spite of these results, no correlation was found with increasing fibrosis, suggesting that a direct role for inflammation in pulmonary fibrosis is unlikely. The data suggest, in the first hand, that fenugreek’s polyphenol has a potent antioxidant activity and therefore has a potent anti-inflammatory activity against bleomycin induced lung fibrosis model in rats, and in the second hand, they confirm that besides inflammation, other factors probably interfere in the pathogenesis of pulmonary fibrosis.

Key words: Lung fibrosis, fenugreek, polyphenols, bleomycin®, oxidative stress, total antioxidant status (TAS), malondialdehyde (MDA), transforming growth factor-beta (TGFβ), rats.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease of unknown cause, and poor prognosis (Psathakis et al., 2006), which is characterized by lesions of the alveolar septa, fibroblaste and myofibroblast proliferation in lung parenchyma, abnormal reepithelisation, and excessive extracellular matrix macromolecule deposition (Manoury et al., 2005). Current treatment includes corticosteroids and cytotoxic agents such as cyclophosphamide or azathioprine. The response rate of corticosteroids treatment in patients with IPF has been...
shown to be 10 to 30% (Iraz et al., 2006). An oxidant/antioxidant imbalance in the lower respiratory tract has been proposed as one of the mechanism of the lung injury in patients with IPF (Rahman et al., 1999), and many studies have identified several potential mechanisms through which the presence of oxidative stress in the lung can lead to increased inflammation and fibrosis (Corrine and Oury, 2010). Therefore, novel therapeutic agents with improved efficacy are needed.

Bleomycin is an antineoplastic agent used in the chemotherapy of different types of cancer that produces its antineoplastic effect by causing oxidant damage to DNA. This toxic effect has been utilized advantageously in experimental models to cause lung injury leading to oxidant induced inflammatory and fibrotic lesions in the lung interstitium of various animal species (Goto et al., 2004). This animal model of pulmonary fibrosis resembles that seen in humans and it is useful to assess the effects of potential therapeutic agents including antioxidants (Serrano-Mollar et al., 2003). Recent studies suggested that many promising agents against idiopathic pulmonary fibrosis include antioxidants. In recent years, a large effort has been taken to explore the possibility of using natural resources to delay the onset and progression of fibrosis. A great number of medicinal plants and their formulations are reported to possess antioxidant properties (Gupta et al., 2009). This property is due to polyphenols, a group of structurally heterogeneous compounds, which have been widely used in phytotherapy for a long time (Hennebelle et al., 2004).

Fenugreek, Trigonella foenum-graecum Linnaeus 1753, which belongs to the family of Fabaceae, is widespread around the Mediterranean basin and on the southern coasts of the Black Sea. The existence of this plant is very ancient and it has been used, since a long time ago, in India, China, Middle East, Egypt and Ethiopia. The species is extensively cultivated in most regions of the world for example, North Africa (Tunisia), Ukraine, India, and China (Ghedira et al., 2010). Fenugreek seeds have been documented for their multiple pharmacological activities including antioxidation (Ahmadiani et al., 2001), fenugreek seed polyphenols prevented oxidative hemolysis and lipid peroxidation induced by H2O2 in vitro in human erythrocyte (Kaviarsan et al., 2004). Moreover, it was demonstrated that the supplementation of fenugreek seed powder in the diet leads to a reduction in biomarkers of oxidative damage in alloxan-diabetic rats (Ravikumar and Anuradha, 1999). It was also showed that the polyphenolic extract of fenugreek seeds has an antioxidation activity in vitro (Kaviarasran et al., 2007), protective action against alcohol-induced protein and lipid damage in rat liver (Kaviarasran et al., 2008) and that it prevents ethanol induced apoptosis in Chang liver cells (Kaviarasran et al., 2006). However, in spite of the therapeutic importance of fenugreek, no study was carried about its effects on pulmonary fibrosis. Within this context, the present study was designed and conducted to investigate the effect of TFG seed extract on experimental pulmonary fibrosis in Wistar rats. Histological and immunohistochemical analyses of the lung were also done to confirm the obtained results.

MATERIALS AND METHODS

Animals

Healthy male albino Wistar rats weighing 233±35 g, purchased from Tunis Pasteur Institute, were used during the present study. Animals were housed in polypropylene rat cages in an animal room, at the Faculty of Medicine of Tunis, with controlled temperature (24±2°C). A 12 h light/dark cycle was maintained and the rats had free access to water and food ad libitum. The animals were cared according to the principles of the International Council of Laboratory Animal Science (ICLAS).

Preparation of plant extract

A fenugreek seed sample was supplied by the Laboratory of Foragers Production, local variety. Extraction was carried out according to the method procedure adopted by Fattouch et al. (2007), with little modifications. Fenugreek seeds (10 g) were finely powdered and mixed with 100 ml of cold acetone/water (70:30 v/v) during three minutes. The mixture was sonicated for 20 min and centrifuged at 10000 rpm, for 15 min at room temperature. The supernatants were collected, pooled, and concentrated to dryness under reduced pressure (40°C) using a rotavap. In order to prevent the oxidation of polyphenols, extraction was achieved rapidly and the extracts were immediately used.

Determination of total phenolic content (TPC)

TPC in the extract of fenugreek seeds was estimated spectrophotometrically by the method of Singleton and Rossi (1965) using the Follin-Ciocicateu’s phenol reagent (Singleton and Rossi, 1965), with minor modifications: each 25, 50 and 100 µl of phenolic extract was oxidized with 400 µl of Follin-Ciocateu 10%. 2 to 5 min later, 500 µl of sodium carbonate (7.5%) were added to neutralise the reaction. After one hour of incubation at room temperature in dark, the absorbance was measured at 725 nm on UV-VIS spectrophotometer. The polyphenolic content was expressed in mg of gallic acid equivalents (GAE) per gram of dry seed, using a standard curve generated with gallic acid.

Free radical scavenging (FRS) activity

The antioxidant activity of the fenugreek phenolic extract was determined using the Trolox equivalent antioxidant capacity (TEAC), which evaluates the scavenging of the free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) relative to trolox, a water-soluble vitamin E analogue. An aliquot (25 µl) of sample solution was added to 1 ml of 40 µM methanolic solution of DPPH. The mixture was shaken vigorously and left to stand for 1 h at room temperature in the dark. Methanol was used as a blank solution, and DPPH solution without any sample extract served as control. The absorbance of the resulting solution was measured at 517 nm. The trolox equivalent antioxidant capacity (TEAC) values were calculated from the equation determined from linear regression after plotting known solutions of trolox with different concentrations.

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anesthetized rat was immediately suspended from a g allows. Pentobarbital sodium solution (Sandoz laboratory, France). Each underwent anesthesia by intraperitoneal injection of 100 µg/g of Pentobarbital sodium solution (Sandoz laboratory, France). Each anesthetized rat was immediately suspended from a gallow. Induction of fibrosis was done by an intra-tracheal injection of 4 mg/kg body weight of bleomycin sulfate solution (Bleomycin®, Laboratories Aventis, France). The day of intratracheal injection with BLM was designated day 0.

Animal model of bleomycin-induced lung fibrosis

The bleomycin rat model of lung fibrosis was used. All rats underwent anesthesia by intraperitoneal injection of 100 µg/g of pentobarbital sodium solution (Sandoz laboratory, France). Each anesthetized rat was immediately suspended from a gallow. Induction of fibrosis was done by an intra-tracheal injection of 4 mg/kg body weight of bleomycin sulfate solution (Bleomycin®, Laboratories Aventis, France). The day of intratracheal injection with BLM was designated day 0.

Study design

Three days after the induction of fibrosis, the animals were divided randomly in four groups (n = 10 in each group): the first group was given fenugreek seed polyphenol extract (FSPE) which was administered orally at a dose of 200 mg kg$^{-1}$ day$^{-1}$, approximately equivalent to 6.5 ml kg$^{-1}$day$^{-1}$, by using intragastric intubation. The second group received fenugreek seeds powder mixed with the commercial diet at levels of up to 20%. The third group received an equal volume of sterilized distilled water, also by daily gavages (6.5 ml kg$^{-1}$ day$^{-1}$). As for the fourth group (Sham), it did not receive any treatment and had free access to food and water. The total experimental duration was two weeks. The experimental design is depicted in Table 1.

Sample collection and analytical procedures

After 15 days of treatment, animals were anesthetized with the same procedure described during induction of fibrosis by injecting intraperitoneal 100 µg/g of pentobarbital sodium solution. Blood samples were obtained from rats by cardiac puncture using evacuated tubes with heparin as anticoagulant (for MDA determination) and without heparin (for TAS determination). All tubes for plasma collection were immediately placed on ice, and within 2 h of bleeding, 500 µl of each sample was centrifuged at 2500 rpm for 10 min at 4°C; the plasma was stored at −80°C until analysis.

Table 1. Experimental design.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment and duration</th>
<th>Day 3 – Day 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FSPE (200 mg kg$^{-1}$ day$^{-1}$ ~ 6.5 ml kg$^{-1}$ day$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>FPS up to 20%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Water (6.5 ml kg$^{-1}$ day$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Free access to water and commercial diet</td>
<td></td>
</tr>
</tbody>
</table>

Lipid peroxidation

Malondialdehyde (MDA), a reactive carbonyl compound formed upon the decomposition of polyunsaturated fatty acid peroxides (Esterbauer et al. 1991), was measured as an indicator of oxidative stress in plasma using the method of Okawa et al. (1979) and Bueje and Aust (1978), with little modifications: 250 µl of plasma was dissolved in PBS (100 µl) and TCA-BHT (250 µl). After centrifugation at 1000 rpm during 10 min, the supernatants were reprimed in HCl and TRIS-TBA. After incubation at 80°C for 10 min, the absorbance was measured at 532 nm using UV-VIS spectrophotometer. The levels of MDA were expressed as nmol/mg protein ($\varepsilon_{532} = 1.56 \times 10^5 M^{-1} cm^{-1}$). Protein level in plasma was determined by means of a colorimetric assay kit specific for total protein using a Biuret method, supplied by Bimaghreb, Tunisia. The levels of protein were expressed in g/L.

Total antioxidant status (TAS)

Serum TAS was measured using the kit supplied by Randox Laboratories Ltd. ABTS® (100 µl), which is based on the incubation of 2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate] with a peroxydase (metamyoglobin) and H$_2$O$_2$ to produce the radical cation ABTS$^+$.

Histological and immunohistochemistry analysis

For histological studies, the lungs were perfused through their main bronchus with fixative solution (10% neutral-buffered formalin), immersed in the fixative for 24 h, and the blocks were taken thereafter. Tissue blocks were placed in formalin dehydrated in a graded series of ethanol, embedded in paraffin, cut into 4 mm thick serial sections, and stained with haematoxylin-eosin (H&E) to identify the inflammatory cells of Masson’s trichrome for collagen deposition. Histological grading of lesions was performed using a blinded semi quantitative scoring system for extent and severity of inflammation and fibrosis in lung parenchyma. The severity of inflammation was estimated using the semi quantitative grading system which considers the following categories: Grade 0 = "absence of inflammation", Grade 1 = "minimal inflammation", Grade 2 = "minimal to moderate inflammation", Grade 3 = "moderate inflammation with thickening of alveolar walls", Grade 4 = "moderate to severe inflammation" and Grade 5 = "severe inflammation with presence of follicles which replace the parenchyma". The severity of interstitial fibrosis was also determined using the semi quantitative grading system, described by Ashcroft et al. (1988). The entire lung section was observed at a ×100 magnification and a score ranging from 0 (normal lung) to 8 (total fibrosis) was assigned. The adopted categories of grading

(0.02 to 0.8 mM). The antiradical activity was also expressed as the inhibition percentage and was calculated using the following formula:

% radical scavenging activity = (control OD - sample OD/control OD) × 100.
Table 2. Means ± SD of malondialdehyde (MDA) and total antioxidant status (TAS) in the treated groups of rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/mg protein)</th>
<th>TAS (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.280 ± 0.053</td>
<td>0.888 ± 0.086</td>
</tr>
<tr>
<td>2</td>
<td>0.205 ± 0.031</td>
<td>0.695 ± 0.086</td>
</tr>
<tr>
<td>3</td>
<td>0.434 ± 0.043</td>
<td>0.345 ± 0.043</td>
</tr>
<tr>
<td>4</td>
<td>0.417 ± 0.034</td>
<td>0.561 ± 0.050</td>
</tr>
<tr>
<td>Total</td>
<td>0.336 ± 0.105</td>
<td>0.607 ± 0.214</td>
</tr>
</tbody>
</table>

pulmonary fibrosis were as follows: Grade 0 = "normal lung", Grade 1 = "minimal fibrous thickening of alveolar or bronchial walls", Grades 2 to 3 = "moderate thickening of walls without obvious damage to lung architecture", Grades 4 to 5 = "increased fibrosis with definite damage to lung architecture and formation of fibrous bands or small fibrous mass", Grades 6 to 7 = "severe distortion of structure and large fibrous areas", "honeycomb lung" was placed in this category; Grade 8 = "total fibrotic obliteration of the field". The mean score of all fields was taken as the fibrosis score of that lung section.

The immunohistochemical studies were performed on one representative block from each case. Sections of 3 to 4 µm were deparaffinized with xylem and ethanol. Endogenous peroxides activity was blocked with 3% hydrogen peroxide for 10 min. Microwave epitope retrieval was used. Immunohistochemical analysis was performed using TGFβ antibody (R&D System Laboratories, France). The density of TGFβ in lung tissue was scored on a scale ranging from 0 to 3: 0 = "absent", 1 = "low", 2 = "medium", and 3 = "important". Micrographics were obtained by camera Nikon Cooplix 4500.

Statistical analysis

Values were reported as mean ± SD. Data comparison among the studied groups were analysed by one-way ANOVA and Tukey POST-HOC comparison tests. This was done using the software SPSS 11.5.

RESULTS

Acute toxicity in rats

Rats administrated FPS did not develop any clinical signs of toxicity either immediately or during treatment and no mortality occurred.

Total phenolic content (TPC) and free radical scavenging (FRS) activity

The antioxidant activity of fruits and vegetables is generally positively correlated with their content of polyphenols; but the extraction of phenolic compounds in plant material is influenced by their chemical nature, the extraction method, time and storage conditions as well as the presence of interfering substances (Prior et al., 2005). During the present study, the total phenolic content of the extract was estimated to be 15.180±1.7 mg GAE/gDW from three determinations. The DPPH antioxidant assay is best on the ability of 1-1-diphenyl-2- picrylhydrazyl, a stable free radical to decolorize in the presence of antioxidants. The DPPH free radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance (Siddiqua et al., 2010). As for percentage of DPPH scavenging activity of our extract, it was measured to be 74.71% corresponding to 73.5 mM TEAC/gDW.

Lipid peroxidation and total antioxidant status (TAS)

Taken together, MDA and TAS are two parameters that could provide complementary information about the oxidant-antioxidant balance. The TAS and MDA results are given in Table 2. An extent of lipid peroxidation was indicated by the level of plasma MDA, measured for the four treated groups. The ANOVA, conducted based on MDA data, showed that the four groups are significantly different ($F = 56.150; p ≤ 0.0001$). In fact, MDA level in plasma of group 3, increased significantly compared with the control group (4).

The MDA levels in the group 1 (0.281 ± 0.0537 nmol/mg protein) and group 2 (0.206 ± 0.0316 nmol/mg protein) were found to be lower than that in the third group. This was confirmed, based on Tukey-HSD test, by the results of Post-Hoc comparison which allowed the classification of the four groups of rats in three clusters that are significantly different: the first two clusters were represented, each one, by the rat groups 1 and 2. While the third clusters consisted of the two last rat groups (3 and 4) (Table 3).

In contrast to the MDA results, TAS increased in the serum of rats treated with FSPE (0.888 ± 0.0868), compared to the rats treated with FPS (0.696 ± 0.0869). The latter values differed significantly with that in serum TAS of untreated rats. This can also confirmed by ANOVA which showed that the four treated groups of rats are different in terms of TAS ($F = 87.359; p ≤ 0.0001$). In addition, the results of Post-Hoc comparison, based on Tukey HSD test, showed the existence of four clusters
Table 3. Post-Hoc comparison (based on Tukey HSD test), of MDA and TAS between the four treated groups of rats (A, B, C, and D are the clusters considered by the analysis).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>Subsets for alpha = 0.05</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>MDA</td>
<td>2</td>
<td>0.20563</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.28071</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.43411</td>
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<td></td>
<td>3</td>
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<tr>
<td>Significance</td>
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<tr>
<th>Groups</th>
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<tr>
<td></td>
<td>A</td>
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<tr>
<td>TAS</td>
<td>3</td>
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<td></td>
<td>4</td>
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<td>2</td>
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<td></td>
<td>1</td>
</tr>
<tr>
<td>Significance</td>
<td>1</td>
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</table>

Table 4. Means ± SD of inflammatory score and fibrosis score in the treated groups of rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Inflammatory score</th>
<th>Fibrosis score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.29 ± 0.49</td>
<td>4.29 ± 1.25</td>
</tr>
<tr>
<td>2</td>
<td>4.29 ± 0.76</td>
<td>4.71 ± 0.76</td>
</tr>
<tr>
<td>3</td>
<td>4.70 ± 0.48</td>
<td>5.80 ± 1.03</td>
</tr>
<tr>
<td>4</td>
<td>5.00 ± 0.00</td>
<td>5.83 ± 1.33</td>
</tr>
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</table>

significantly different; each cluster is represented by one group of rat (Table 3).

Histological and Immunohistochemical analyses

The histological analysis showed the existence of a significant difference between the four treated groups ($F = 14.587; p \leq 0.001$). In fact, the rats of groups 1 and 2 presented the lowest values of inflammatory index, in comparison with the other groups (Table 4). Besides the results of Post-Hoc comparison, which allowed the two clusters to be distinguished as significantly different, the first subset was represented by only group 1; while the second was formed by the three other groups that is, 2, 3 and 4 (Table 5). It is worth noting that FSPE appeared to be more effective in reducing inflammation ($3.29 \pm 0.49$) than the group treated with FPS ($4.29 \pm 0.76$). Otherwise, the fibrosis score (Table 4) was not found to significantly differ between all groups (ANOVA, $F = 3.742; p = 0.023$). Hence, Tukey HSD classified all treated groups in only one cluster (Table 5).

The immunohistochemical study showed partial effectiveness of FSPE and FSP treatment, especially on the inflammatory infiltrate (bronchiolar and peribronchiolar).

In fact, it induced with treated rats a significant difference in $TGF_{\beta}$ density, compared to the control group (ANOVA: $F = 3.993; p \geq 0.018$ for bronchiolar; $F = 3.785; p \geq 0.022$ for peribronchiolar). However, the treatments with FSPE and FSP did not present any effect on macrophages (ANOVA: $F = 1.280; p \geq 0.302$). The results of immunohistochemical study are presented in Figure 1.

DISCUSSION

Herbal medicine has been used for more than 5000 years. The interest in polyphenols has grown considerably because of their high capacity to trap free radicals associated with different diseases. To our knowledge, this is the first study to evaluate the effect of TFG, in lung on experimental lung fibrosis. Bleomycin is a chemotherapeutic antibiotic produced by bacterium Streptomyces verticillus (Vallyathan and Shi, 1997). It causes inflammatory and fibrotic reactions within a short period of time, and even more intratracheal instillation. This drug, induced pulmonary fibrosis in rats, is widely used as a model exhibiting pathology similar to that found in human IPF. Considering all these points, we picked...
Table 5. Post-Hoc comparison (based on Tukey HSD test), of inflammatory index and fibrosis score between the four treated groups of rats (A and B are the clusters considered by the analysis).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>Subsets for alpha = 0.05</th>
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<tbody>
<tr>
<td></td>
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<td>A</td>
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<tr>
<td>Inflammatory index</td>
<td>1</td>
<td>3.29</td>
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<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
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<th>Groups</th>
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<tr>
<td></td>
<td>A</td>
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<tr>
<td>Inflammatory index</td>
<td>1</td>
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<td></td>
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<td>3</td>
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<td></td>
<td>4</td>
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<tr>
<td>Significance</td>
<td>0.057</td>
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Figure 1. Variability of TGF-β density in peribronchial inflammatory infiltrate, bronchial inflammatory infiltrate, and macrophages, between the four treated groups of rats.

Experimental pulmonary fibrosis induced by bleomycin in wistar rats as a model system of our study. Otherwise, because of the complexity of various natural mixtures of phenolic compounds, many extraction methods have been reported in the literature using different solvents. In the present data, we used a simple aqueous acetone extraction method to prepare a fenugreek seeds polyphenols.

The antioxidant capacity of the fenugreek extract was analyzed using DPPH, a stable free radical having a maximum absorption at 517 nm. The highest antioxidant activity of the acetonic extract (74.71%) could be correlated with the polyphenolic components present in the extract.

The evaluation of oxidative stress can be monitored by several biomarkers. In our study, we choose to measure two parameters: the plasma levels of malondialdehyde (MDA) because of its facile reaction with thiobarbituric acid forming an intensely colored chromogen, and the total antioxidant status (TAS) due to its general idea...
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Figure 2. Histological analysis (H&E) of lung tissue from rat received BLM + water (A), rat received BLM (B), rat received BLM + FSPE (C), and rat received BLM + FPS (D). Sections note intense inflammation (asterisk), disruption of alveolar architecture with presence of lymphoid follicles (arrow) and significant interstitial thickening in untreated group (A and B). A marked regression of inflammation was showed in treated group (C and D). Lung sections were obtained at two weeks post treatment from all rats. One representative example is shown for each group. Original magnification × 200.

Concerning inflammation, the histopathological changes correlated with biochemical changes showed that fenugreek seed have anti-inflammatory effect. In fact, the entire pulmonary parenchyma of untreated groups appeared affected by the inflammatory process: lungs from rats showed marked peribronchiolar and interstitial infiltration which inflammatory cells (predominantly mononuclear cells including macrophages and lymphocytes), extensive thickening of interalveolar septa, interstitial oedema, and increase in interstitial cells (Figures 2A and B). However, panels of treated groups showed alternating zones of normal and inflammatory/fibrosing lung parenchyma. The process of inflammation became patchy and only little zones are affected by inflammation (Figures 2C and D). The highly levels of inflammation in untreated groups confirm the idea that an acute inflammation characterizes the initial reaction of BLM in the lung. Besides, the decrease of lung inflammation in treated groups is a proof that active components concentrating in FSPE and responsible for this effect are certainly a member of polyphenols family. Our results strongly confirm the findings of Ahmadiani et
al. (2001) and Vyas et al. (2008) who reported that fenugreek phenolic extract has a potent anti-inflammatory activity. Within this context, some authors have demonstrated a linear correlation between the content of total phenolic compounds and their antioxidant capacity (Wojdylo et al., 2007). This is possible since a number of antioxidant-nature phytochemicals have been reported to be present in fenugreek seeds (Kaviarasan et al., 2007). These latter include N,N′-dicarbazyl, glycerolmonopalmitate, stearic acid, β-sitosteryl glucopyranoside, ethyl-α-D-glucopyranoside, D-3-O-methylchiroinositol and sucrose (Shang et al., 1998a), gallic acid, o-coumaryl acid, p-coumaric acid, rutin, and caffeic acid (Dixit et al., 2005), vitexin, tricin, naringenin, tricin-7-O-β-D-glucopyranoside and quercetin (Shang et al., 1998a, b). The last compound is one of flavonoids family which has shown a markedly protective action against paraquat induced lung fibrosis (El-Sayed and Rizk, 2009) and several studies have demonstrated that dietary quercetin enhanced the antioxidant defence system by up regulating antioxidant enzymes (Nagata et al., 1999).

Histologically, lung sections with H&E showed loss of normal pulmonary architecture. The BLM-water and BLM groups marked histopathologic changes, such as inflammatory cell infiltration, large fibrous areas, collapsed alveolar spaces (Figures 2A and B). However, in rat treated with FPS, we noted a marked regression of inflammation (Figures 2C and D). In spite of this result, no significant effect on pulmonary fibrosis was marked in groups having received FSPE and FSP. In the sections stained with Masson’s trichrome, this was used as an index of collagen deposition and with which muscle and cells are stained red, nuclei are stained black and collagens are stained green. As a result, apparent decrease in trichrome staining was seen (Figures 3A, B, C and D). The localisation of collagen deposition may be due to the route of administration of BLM (Hay et al., 1991). Considered together, these results suggest that fenugreek seeds especially polyphenols have an effect

Figure 3. Masson trichrome: section of lung tissue from rat received BLM + water (A), rat received BLM (B), rat received BLM + FSPE (C), and rat received BLM + FPS (D). Sections note inter alveolar oedema (asterisk), inflammatory infiltrate of septa (arrow). Lung sections were obtained at two weeks post treatment from all rats. One representative example is shown for each group. Original magnification × 100.
on inflammation, but no major effect on structural disorganisation resulting from BLM and indicate that the inflammatory response and the fibrotic response can be dissociated. Transforming growth factor β (TGF-β) is considered to be the most potent and ubiquitous profibrogenic cytokine (Liu and Gaston, 2010). Previous studies have shown that TGF-β, if released soon after injury, acts primarily as a proinflammatory molecule because it has potent chemotactic effects on inflammatory cells. Therefore, consistently elevated levels of TGF-β in the lung may serve as a stimulus for myofibroblast activation and production of extracellular matrix (Kinnula et al., 2005). ROS/RNS play important roles in the development of fibrosis and in TGF-β-mediated fibrogenesis. The molecular mechanism by which ROS/RNS mediate TGF-β induced fibrosis, however, remains undetermined (Wojdylo et al., 2007). In our study, a pattern of immunohistochimical straitening suggested that macrophages are stained in all groups of rats. Immunostaining was concentrated on the interstitium of the thickened alveolar septa (Figures 4A, B, C and D). Moreover, this work showed no significant effect of fenugreek to reduce fibrosis which is consistent with the literature data currently suggesting that inflammation is not the only factor that leads to the fibrotic process. Idiopathic pulmonary fibrosis is a complex disorder, and no unifying hypothesis explaining all the abnormalities has been identified at present. Our study demonstrated that TGF-β, widely expressed in normal lungs, increased in areas of active fibrosis in bleomycin induced pulmonary fibrosis.

Many studies have been performed for treatment or prevention of pulmonary fibrosis. However, no effective treatment has been found yet. Despite that medicinal
plants know a renewed interest, there are not many published studies that evaluate the effect of plants on this disease, which is the most deadly diffuse interstitial pneumonitis. Among the plants that were used to evaluate their effects on pulmonary fibrosis: green tea (El-Sayed and Rizk, 2009), curcuma (Punithavathi et al., 2000), grape seeds (Hemmatia et al., 2006) and Houtthuynia cordata (Hc), a vegetable commonly consumed in Taiwan (Ng et al., 2007). Apart from green tea, which is used in a model of pulmonary fibrosis induced by Parafquat, the common point of the three other works with ours is that they all try to exploit the anti-inflammatory, antioxidant property of these plants extracts on a model of fibrosis induced by BLM in rats by orally administration. The present study noticed that all these studies have used doses higher than ours: curcumin (300 mg/kg b.w), Hc extract (1 g/kg b.w) and gape seeds extract (100, 200, 400 mg/kg b.w, but the last dose in this study is most effective). Considering all these studies, we can conclude that the limited effect of fenugreek seeds and their extracts on experimental fibrosis in our study may be due to the dose. Further dose-effect studies are necessary to better understand this aspect.

Conclusion

The present work aimed to enlighten the relationship between redox-balance and inflammatory mechanisms in experimental pulmonary fibrosis. The pulmonary system is particularly vulnerable to ROS induced injury because of its continuous exposure to toxic pollutants from a wide variety of sources in the ambient air. A powder supplementation and an aqueous acetonic extract of fenugreek seeds was delaminated for its antioxidant and anti-inflammatory properties of germinated fenugreek seeds. Phytother. Res., 19: 977-983.

Abbreviations: IPF, Idiopathic pulmonary fibrosis; ROS, reactive oxygen species; RNS, reactive nitrogen species; TFG, Trigonella foenum-graecum; FSPE, fenugreek seed polyphenol extract; TAS, total antioxidant status; OD, optic density; GAE, gallic acid equivalents; PBS, phosphate buffered saline; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; BHT, butylhydroxy tolulene; BLM, bleomycin; DPPH, 2,2-diphenyl-1-picrylhydrazy; TEAC, trolox equivalent antioxidant capacity; MDA, malonaldehyde; TBA, thioarbituric acid; TGFβ, transforming growth factor-beta; ABTS®, 2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate]; SD, standard deviation; FPS, fenugreek powder supplementation.

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