Effect of hydroalcoholic and aqueous extracts of *Dracocephalum kotschyi* on bleomycin induced pulmonary fibrosis

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**Implication for health policy/practice/research/medical education:** *Dracocephalum kotschyi* extracts displayed systemic anti-inflammatory and anti-fibrotic activities, and reduced biochemical and histopathological indices of pulmonary inflammation and fibrosis. Therefore, it might be useful for prevention of idiopathic pulmonary fibrosis.

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**Introduction:** Idiopathic pulmonary fibrosis is a progressive and usually fatal chronic interstitial lung disease with very limited treatment options (1). Fibrosis occurs due to the large accumulation of extracellular matrix proteins. These proteins include collagen, elastic fibers, fibronectin and laminin, among which collagen is the most abundant (2). The disease is characterized by abnormal activity of epithelial cells, in which over excretion of mediators causes overcrowding of fibroblasts followed by excessive accumulation of extracellular matrix and eventually destruction of the lung tissue (2). Acute distress syndrome
caused by acute lung injures and traumas results in chronic interstitial pulmonary fibrosis, which is characterized by progression of fibrosis and collagen deposition (3). The pathogenetic mechanism of fibrosis is not clear (4). Studies have shown that aging, smoking, environmental and occupational factors are important known risk factors (5,6). Viral infections, genetic factors, exposure to paraquat, toluene, certain drugs such as methotrexate, amiodarone and bleomycin have also been identified as contributing factors (6,7). With the increasing prevalence of cancers, especially solid tumors, bleomycin is used as one of the drugs in the chemotherapy regimen (8). The pulmonary fibrosis complication of this drug is high. Glucocorticoids and immunosuppressive drugs are common treatment for idiopathic pulmonary fibrosis (9). However, treatment of fibrosis with immunosuppressive drugs is not satisfactory and in most cases accompanied with serious adverse effects (10). Considering the cost of current drug treatments and their side effects, alternative and more effective therapies are required. One approach is use of herbal medicine with known active constituents with anti-fibrosis activity (11).

Dracocephalum kotschyi Boiss is one of the native plants of Iran locally known as Zarringiah (12). D. kotschyi belongs to Lamiaceae family and contains numerous flavonoids constituents with antioxidant properties (13). In traditional medicine, this herb has been used as a medicinal plant for the treatment of rheumatism and gastrointestinal problems (13). Several separate researches have reported that D. kotschyi possess immunomodulatory, anticancer, anti-hyperlipidemia, antidiabetic, anti-inflammatory and antispasmodic properties (14-20). D. kotschyi is enriched in various constituents including essential oils and flavonoids (21,22). The main substances found in D. kotschyi essential oil include α-pinene, neral, geraniol, α-citral, limonene, cyclononadiene, terpinene-4-ol linalool, carveol, myrcene, germacrene-D, isopinocarveol, and α-terpineol (23). The constituents of hydroalcoholic extract, include calycoperin, xanthomicroil, isokaempferide, luteolin, apigenin, luteolin-7-O-beta-D-glucopyranoside, lutcolin-3'-O-beta-D-glucuronicide, apigenin-4'-O-beta-D-glucopyranoside, acacetin-7-O-beta-D-glucopyranoside and rosmaninic acid (22). Both the essential oil and hydroalcoholic extract of D. kotschyi have strong spasmylic and anti-inflammatory activities (24,25). Limonene and α-terpineol are responsible for the anti-nociceptive properties of the essential oil of D. kotschyi leaf (26). Also, methoxylated flavones such as apigenin, luteolin, isokaempferid, cirsimaritin, calycoperin, penduletin and xanthomicroil have been reported to be responsible for its anticancer effects (15,27-30). Luteolin has multiple biological properties such as anti-inflammatory, antioxidant and anticancer activities (31-33). In animal studies, luteolin clearly inhibited the growth and proliferation of lung fibroblasts (34) and demonstrated strong anti-fibrotic activity both in vitro and in vivo (35). Apigenin has anti-inflammatory and antioxidant properties (20). It also has cytostatic and cytoprotective activities (29,30). In a separate study, the anti-inflammatory and anti-fibrotic effects of apigenin on fibrotic lungs induced by bleomycin were demonstrated (36). As it was mentioned above, apigenin and luteolin are abundant flavonoids compounds exist in D. kotschyi (22). D. kotschyi extracts have been reported to have bronchodilatory effects on rabbit tracheal (37). In addition to anti-inflammatory and anti-fibrotic activities both apigenin and luteolin also have bronchodilatory properties (37). Thus, it is likely that D. kotschyi extract has anti-inflammatory and anti-fibrotic activities on pulmonary fibrosis. Therefore, the main objective of this research was to investigate anti-inflammatory and anti-fibrotic effects of hydroalcoholic and aqueous extracts of D. kotschyi in animal models.

Methods and Materials

Extract preparations

Zarringiah was collected from cultivated farm belong to Rahnama Kesht Pertikan Company located in the Shahankoo in Fereidounshahr (Isfahan-Iran). The plant was identified by the botanist Mr. Mohammad Isfa from Department of Natural Resources of Isfahan province as D. kotschyi. A voucher of the plant has been deposited in School of Pharmacy and Pharmaceutical Sciences herbarium (No: 1519). The leafy branches of the plant were dried in shade and grinned into fine powder with a grinder (Keep, Korea). Plant extraction was performed by decoction and maceration techniques (38,39).

For preparation of aqueous extract, in a large baker 750 mL of water was added into 30 g of dried powder and brought to boil and then simmer for up to 1 hour until the volume was reduced to one third. The whole content was strained through Buchner funnel and then the collected liquid was concentrated by rotary apparatus (Heidolph, Germany). The concentrated extract was then freeze dried and the extract yield was calculated.

For preparation of hydroalcoholic extract the dried powder was moist with 70% ethanol and kept in a sealed container for 2 hours. The moistened plant powder was transferred to percolorator and immersed in 70% ethanol (1:8 ratio) for three days accompanied by gentle shaking. After three days, the extract aliquant was removed. This process was repeated two more times by adding 70% ethanol to the percolorator. The collected extract was concentrated by rotary apparatus.

Drug and solutions

Lyophilised bleomycin sulphate (15 000 IU equivalent to a standardised biological activity of 15 mg bleomycin) (Bleo-Cell vial, Germany) was dissolved in 0.9% normal saline as 5000 IU/mL stock solution for immediate use.
Formaldehyde was diluted with water to produce 10% solution. Hydroalcoholic extract of *D. kotschyi* was dissolved in 20% dimethyl sulfoxide (DMSO) in normal saline to give 8 mg/mL stock solution. Aqueous extract and pirfenidone were prepared in normal saline as 8 mg/mL and 2 mg/mL stock solutions, respectively. Further dilution was made with normal saline.

**Experimental procedure**

Fibrosis was induced in rat by intra-tracheal instillation of bleomycin. Adult and healthy male Wistar rats (180-200 g) were used for this experimental study. The animals were purchased from School of Pharmacy and Pharmaceutical Sciences animal house. Animals were anesthetized by isoflurane in a glass container (500 mL) under laboratory hood. A piece of cotton wood was soaked in isoflurane solution (30% v/v isoflurane in propylene glycol) and placed inside the container. When the rat was tilted on its back and did not turned on its feet consider as anesthetic cut point. The animal was then removed and placed on its back and its mouth was opened up by hanging upper canine teeth with a thread. Bleomycin (200 IU/kg) was instilled inside the trachea via a metal feeding tube. All the drugs were given orally by feeding tube every day for 4 weeks. The test groups were treated with either hydroalcoholic or aqueous extract of *D. kotschyi* (20 mg/kg, 40 mg/kg & 80 mg/kg). The control groups were treated either with normal saline or DMSO as appropriate (negative group). The positive group was treated with standard drug pirfenidone (100 mg/kg). In the sham groups, one group received no treatment at all, while other group received intra-tracheal instillation of normal saline without other treatment. At least 6 rats were used in each group. The animals were kept in a separate room in the animal house with free access to food and water at room temperature. Prior and at the end of the study all the animals were weighed.

On 28th day, all animals were sacrificed with CO₂. Then the animal's thoracic cage was opened up and both lung tissues were carefully dissected out and weighed. The lung tissues were washed with normal saline and a lob was placed in formaldehyde for histopathological examination. The other lob was frozen at -80°C for biochemical assessment. For histopathological evaluation, lung tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm thickness, and stained with Masson's trichrome and hematoxylin-eosin staining for light microscopic examination. The parameters that were assessed in histopathological sections consisted of vascular congestion, hemorrhage, interstitial edema, alveolar structural disturbance, infiltration of inflammatory cells and fibrosis. Description and scoring of histopathological lesions and evaluation of fibrosis changes were carried out as previously described by Greco et al (40) with some modifications (41). Briefly, 10 fields for each lung section were systematically examined using a x10 objective and each field was scored using the following grading scheme: grade 0 for normal tissue and grades 1-4 for the presence of pulmonary inflammation and fibrosis. The severity of lesions was graded as 1 (mild), 2 (moderate), 3 (severe) and 4 (severe inflammation accompanied by total distortion of the structure). The extent of lesions was graded as 1 (>10% of the slide), 2 (10%-40%), 3 (40%-70%), and 4 (<70%) of tissue affected. Fields predominantly occupied by portions of large bronchi or vessels were not counted.

**Biochemical assessment**

In the biochemical assessment hydroxyproline was measured as an index of tissue collagen contents and malondialdehyde was assessed as an indication of lipid peroxidation factors. Hydroxyproline was measured by a standard kit (KHPA 96, Kiazist Hamedan Company, Iran) as explained in its protocol manual. Briefly, a small piece of defreeze tissue (140 to 280 mg) was homogenized and digested in strong acid (12M HCl) and after oxidative reaction with chromogen the supernatant had an orange-purple color. Pure hydroxyproline was used for construction of standard curve and light absorption was read at 550 nm wavelength with Elisa (Elisa reader BioTek).

Malondialdehyde content of the tissue was measure by colorimetry-fluorimetry lipid peroxidation kit (KMDA-96, Kiazist Hamedan Company, Iran). Briefly, malondialdehyde was reacted with thiobarbituric acid to form a complex which gives orange color. Light absorption was read at 550 nm wavelength with Elisa colorimetry apparatus.

**Data assessment and statistical analysis**

Tissue hydroxyproline and malondialdehyde was measured from constructed standard curves and expressed as mg/g and nM/g tissue, respectively. Data values were expressed as mean ± SEM (standard error of mean). For statistical comparison Student's t test was used for parametric data. For non-parametric data Kruskal-Wallis test was used. GraphPad Prism computer program software was used for statistical analysis. *P* values less than 0.05 was considered statistically significant.

**Results**

Bleomycin was used as a tool to induce pulmonary fibrosis. In microscopic examination lung tissues in the control rats (without bleomycin) showed normal bronchial and alveolar spaces and normal thickening of alveolar septa (*Figure 1*). The main histopathologic findings in bleomycin group were disturbance of alveolar structure, severe necrosis and epithelial degeneration of alveolar walls.
with extensive cellular thickening of interalveolar septa, vascular congestion, interstitial edema, severe infiltration of inflammatory cells (predominantly mononuclear cells including macrophages and lymphocytes and less neutrophils) in the interstitial and peribronchiolar area along with many collagen bundles had grown into the interstitial area (Figure 1).

The pattern of lesions in the treated groups with hydroalcoholic and aqueous extracts of *D. kotschyi* were clearly resolved. The extent and severity of the lesions, particularly the necrosis development, inflammation and fibrosis, were markedly less severe in compared with bleomycin treated group (Figures 2 and 3). In comparison between treated groups, the most therapeutic effects were observed with the administration of hydroalcoholic extract of *D. kotschyi*. Generally, high dose of the extracts (80 mg/kg) had a more preventive effect on the damaged lungs compared to lower doses (Figures 2 and 3). To confirm the effects of *D. kotschyi* extracts on the lung injuries induced by bleomycin, a semiquantitative score of the severity and extent of inflammation in the lung sections were estimated by numerical scoring (Figure 4). According to the observations made on the Masson's trichrome-stained sections, fibrosis score was markedly increased in the rats treated with bleomycin, but these scores were significantly reduced in the rats treated with different doses of extracts of *D. kotschyi* and pirfenidone (Figure 5). Similarly, the increase in inflammation score induced by bleomycin significantly ameliorated by the treatments.
with pirfenidone and all doses of *D. kotschyi* extracts except with lowest doses of aqueous extract (20 mg/kg). Neither pirfenidone nor *D. kotschyi* extracts doses used in this study provided 100% protection against bleomycin induced inflammation and fibrosis.

Tissue hydroxyproline level was measured as an assessment of collagen infiltration in the lung tissue. Hydroxyproline is one of the constituents of collagen forming main components of the extracellular matrix. This amino acid is exclusively present in collagen, and its measurement directly indicates the amount of collagen in the tissue sample. In the sham group receiving normal saline and those without treatment, there was no significant difference between hydroxyproline contents in the lung tissues. However, in the group, which received bleomycin alone there was a sharp increase in the hydroxyproline contents (Figure 6). Hydroalcoholic extract of *D. kotschyi* in a concentration dependent manner reduced bleomycin induced hydroxyproline deposition (Figure 6). The hydroxyproline levels were reduced by 25%, 39% and 51% with oral doses of 20, 40 and 80 mg/kg, respectively. Oral administration of aqueous extract also in a concentration dependent manner inhibited hydroxyproline production. The percentage reduction in hydroxyproline level was calculated as 17%, 33% and 46% with oral doses of 20, 40 and 80 mg/kg, respectively (Figure 6). In the

![Figure 5. Numerical scoring of the severity and extent of bleomycin induced fibrosis in rat lungs. On microscopic examination each field was scored using the following grading scheme: grade 0 for normal tissue and grades 1-4 for the presence of pulmonary fibrosis. Fibrosis was induced by intratracheal instillation of bleomycin (5 mg/kg). *D. kotschyi* extracts, pirfenidone or vehicle were given orally for 4 weeks. Collected data are expressed as multiplication of severity and extent of inflammation and presented as meansSEM (n=6). Stars show statistically significant difference with the vehicle treated group. Kruskal-Wallis test was used for statistical comparison (**P<0.001).](image)

![Figure 4. Numerical scoring of the severity and extent of bleomycin induced inflammation in rat lungs. On microscopic examination each field was scored using the following grading scheme: grade 0 for normal tissue and grades 1-4 for the presence of pulmonary inflammation. Inflammation was induced by intratracheal instillation of bleomycin (5 mg/kg). *D. kotschyi* extracts, pirfenidone or vehicle were given orally for 4 weeks. Collected data are expressed as multiplication of severity and extent of inflammation and presented as mean ± SEM (n=6). Stars show statistically significant difference with the vehicle treated group. Kruskal-Wallis test was used for statistical comparison (**P<0.001).](image)

![Figure 6. Hydroxyproline level assessments in bleomycin induced fibrosis in the rat lungs. Hydroxyproline level was calculated from constructed standard curves and expressed as mg/g tissue. Fibrosis was induced by intratracheal instillation of bleomycin (5 mg/kg). *D. kotschyi* extracts, pirfenidone or vehicle were given orally for 4 weeks. Collected data are expressed as multiplication of severity and extent of inflammation and presented as mean ± SEM (n=6). Stars show statistically significant difference with the vehicle treated group. Student's t-test was used for statistical comparison (*P<0.05, **P<0.001).](image)
positive control group, pirfenidone (100 mg/kg), the hydroxyproline level was reduced by 55% compared with the control group (Figure 6).

Amount of malondialdehyde content in the lungs represents tissue oxidative stress which is an indicator for tissue inflammation. As shown in Figure 7, a residual level of malondialdehyde content in the lungs of sham group was seen in comparison to the bleomycin treated group. In the group which received normal saline instead of bleomycin there was no significant increase in malondialdehyde level (Figure 7). Daily treatment with D. kotschyi extracts, significantly attenuated malondialdehyde contents due to bleomycin induced inflammation (Figure 7). The inhibitory effect of both hydroalcoholic and aqueous extracts were concentration dependent. For example, the hydroalcoholic extract reduced malondialdehyde contents of the lung tissue by 41%, 48% and 51% with oral doses of 20, 40 and 80 mg/kg, respectively (Figure 7). Similar pattern of inhibition was seen with aqueous extract of D. kotschyi (Figure 7). Pirfenidone (100 mg/kg) inhibited malondialdehyde production by 53% in comparison with the control group (Figure 7).

Discussion
Pulmonary fibrosis develops following increase in collagen deposition and cell proliferation in the interstitial tissues (2). Fibroblasts and myofibroblasts are responsible for collagen production which results in lung functional incapability (3). Management of pulmonary fibrosis proved to be difficult with limited success. In this study bleomycin induced lung injury was used to investigate anti-inflammatory and anti-fibrotic actions of D. kotschyi extract in an animal model. Bleomycin triggers a series of immunological reactions which enhance inflammatory cells mobilization and thereby cause injury to alveolar wall (42). Furthermore, bleomycin provokes fibroblast proliferation, the activity of which, results in increased collagen deposition and fibrosis in the alveolar interstitial region (3). The pathophysiology of pulmonary fibrosis induced by intra-tracheal instillation of bleomycin involves complex interaction among pro-inflammatory factors including inflammatory cytokines interleukin-1b (IL-1b), tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β). Although these factors may play crucial roles in the establishment of pulmonary fibrosis, however, multiple mediators and cell lineages are involved (42). Histological changes observed following intra-tracheal instillation of bleomycin is believed to have close resemblance to chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis (43). Furthermore, pro-inflammatory cytokines have been suggested to be responsible for enhanced responses of airway smooth muscles and it is believed that they play a significant contribution into the over reactive response of airways observed in asthma (43).

Pirfenidone has been introduced as an anti-fibrotic agent (44). Pirfenidone inhibits fibroblast proliferation, reduces extracellular matrix production and prevents TGF-β dependent collagen synthesis (45).

The pathological examinations of the lungs tissues showed that thickness of alveolar wall and development of fibrosis were reduced by co-treatment with D. kotschyi extracts in the bleomycin treated rats. Furthermore, D. kotschyi extracts reduced accumulation of hydroxyproline in the rat lungs in group which received bleomycin. Hydroxyproline is one of the collagen component mainly found in outer cell matrix (46). This amino acid is unique for collagen and its measurement directly represents sample collagen contents (47). In this research maximum increase in hydroxyproline level and malondialdehyde activity in comparison to the vehicle treated group. In the group which received D. kotschyi extracts and bleomycin, there was a substantial reduction in malondialdehyde activity in comparison to vehicle treated groups. Malondialdehyde is one of the factors involved in lipid peroxidation and cause direct oxidative damage to unsaturated fatty acid in the cells (48). Malondialdehyde is considered as a reliable marker...
for assessment of inflammation in the injured tissues (48). Therefore, these results not only indicate anti-oxidant activity but clearly demonstrate the anti-inflammatory and anti-fibrotic actions of D. kotschyi extracts.

Both hydroalcoholic and aqueous extracts of D. kotschyi demonstrated relatively similar preventive effect against bleomycin elicted pulmonary fibrosis. This resemblance could be due to presence of common components. D. kotschyi extracts are enriched in flavonoids which have potential anti-inflammatory properties (19, 22). Flavonoids mainly exist in glycoside forms which are water soluble and found in both aqueous and hydroalcoholic extracts. In this research both extracts were administered orally and their anti-inflammatory effects on the lungs indicate good oral absorption of active components. The pharmacological activity of flavonoids is mainly seen with their aglycone forms. However, it has been reported that in the gastrointestinal tract the sugar moiety is removed and aglycone form of flavonoids is released (49). There are substantial reports that indicate some active flavonoids are inhibitors of pro-inflammatory cytokine induced chemokine expression (50). Apigenin and luteolin are two known active flavonoids components that have been identified in the D. kotschyi extracts (22). Both these flavonoids have been reported to prevent oxidative stress and inhibit expression of inflammatory factors involved in bleomycin induced fibrosis (33-36). Furthermore, apigenin and luteolin are reported as inhibitors of TGF-β, which can ameliorate features of bleomycin induced lung fibrosis (35,51). Pharmacological actions of apigenin and luteolin have close resemblance to D. kotschyi extracts. Hence, it is likely that flavonoids could be responsible for the anti-inflammatory and anti-fibrotic activities seen with D. kotschyi extracts. Nevertheless, contribution of other component can’t be excluded and requires further investigations.

Present study shows that D. kotschyi extracts prevent collagen deposition, inhibit lipid peroxidation and malondialdehyde activity and thereby reduce pulmonary fibrosis. Anti-inflammatory actions of D. kotschyi extracts on lungs tissue, combined with their bronchodilatory effects on airways smooth muscles (37), make them suitable remedies for treatment of bronchitis asthma.

Conclusion

In animal models, D. kotschyi extracts displayed systemic anti-inflammatory and anti-fibrotic activities and reduced biochemical and histopathological indices of pulmonary fibrosis. Therefore, it is suggested to be used alongside of chemotherapy treatment with anti-cancer agents such as bleomycin in order to prevent development of pulmonary fibrosis. In addition, oral drug preparation from D. kotschyi extracts would have beneficial anti-inflammatory effect in patients with obstructive pulmonary disease and asthma.

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Authors’ contributions
AH was project manager. AH and HS supervised the pharmacological studies. SES supervised extract preparation. ZM was responsible for the experimental work and analysis of data. MH was responsible for histopathological examination of lung tissues. HS was responsible for writing the paper. All authors approved the final manuscript for publication.

Conflict of interests
The authors declare no conflict of interests.

Ethical considerations
Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Isfahan University of Medical Sciences. The project was confirmed by the ethical committee of the university (IR.MUI.RESEARCH.REC.1398.317).

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