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Study of the Combined Impact of Diclofenac and Lemon Balm Extracts on the Reduction of Pulmonary Fibrosis Caused by Bleomycin

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ABSTRACT: Bleomycin antibiotic has the minimal toxic impacts on bone marrow cells due to its anti-cancer activity. Therefore, the blood side effects caused by it are minimal. The main problem by using this drug is pulmonary fibrosis. In the present study, the impact of combination of the plant extracts of lemon, with its antioxidant and anti-cancer activity property, and Diclofenac as an anti-inflammatory on pulmonary fibrosis inhibition resulting from Bleomycin injection has been investigated. In this study, three groups of adult male Wistar rats were selected. Positive control group, negative group, and combined group who received 600 ?g / kg Diclofenac and 75 mg / kg extract of lemon balm simultaneously as a treatment in a 7-days pre-treatment period for 14 days after the prescription of endotracheal Bleomycin. The results show that lung weight, the rate of hydroxyproline, the rate of malondialdehyde in lung and the ratio of lung weight to animal weight on the last day of bleomycin group compared with control group were 65, 73, 129, and 91%, respectively (p <0.05). The increase of these factors in the combined group of diclofenac and lemon balm extracts are 7, 19, 8, and 15%, respectively. According to the results, it can be stated that Diclofenac is a strong inhibitor of cyclooxygenase path that this effect is probably due to the reduction of prostaglandin synthesis. Due to its antioxidant and anti-inflammatory impact, lemon balm herb can inhibit accumulation of collagen.

Keywords: Bleomycin, Pulmonary fibrosis, Diclofenac, Lemon Balm Extracts

INTRODUCTION

Due to minimal toxic effects on bone marrow and hematopoietic system and reducing its immunosuppressive properties, Bleomycin is present in most of the multi-drug chemotherapy regimens of cancer. Unfortunately, following its use in 0 to 40 percent of cases, the side effects of pulmonary fibrosis are fatal in 1% of cases (Thomas and Tedder 2010). Pulmonary fibrosis is a fatal disease that cannot be cured easily. It usually starts with damaged air sacs followed by inflammation and accumulation of collagen and extracellular matrix components in the walls of the air sacs (Tyler and Duane 2014, Luigi *et al.*, 2013). The created damage stimulates the epithelial and endothelial cells, resulting in the accumulation of inflammatory cells in the air sacs. Then, the released oxygen radicals lead to secretion of cytokines. These factors are associated with proliferation of fibroblasts in the interstitial area of the air sacs, the renewal of collagen gene expression, and thus deposition of collagen in the walls of the air sacs (Chen *et al.*, 2006, Kim *et al.*, 2006). As walls between the alveolar are thickened and torn, the effective respiratory spaces disappear. In advanced stages of the disease, including components of this connective tissue, such as collagen and elastin increase. Increased collagen, as the most important component of the connective tissue in the lungs, causes large changes in the structure and function of this vital organ (Rouhani *et al.*, 2009). Many factors are involved in the creation of pulmonary fibrosis, including weakened immune system, active radicals, the presence of molecular oxygen and the presence of bleomycin as a complex with Fe^{2+}. In the process of reaction with DNA in the presence of molecular oxygen, Bleomycin produces active oxygen radicals that attack lung cell membranes and produce lipid peroxide and active radicals active, and this process proceeds in the form of chain reactions. This process is leading to pulmonary fibrosis and the free radicals stimulate the secretion of inflammatory cytokines TNF from lymphocytes and macrophages (Lisa and Fred 2012). Lemon balm is a herb having anti-inflammatory, antimicrobial and antioxidant properties (Birdane *et al.*, 2007, Patora and Klimek 2002).

Antioxidants minimize the destructive effects of free radicals and protect the body cells against the damages caused by these radicals. Lemon balm is a strong collector of active oxygen radicals. Lemon balm leaf has dozens of different chemicals that at least 70 of them have been identified.

Chemical compounds present in the lemon balm herb are divided into four chemical classes: monoterpen, terpene, phenyl and flavonoids. The extract of lemon balm, due to high levels of quercetin, causes resistance against the oxidative damage caused by various factors. Diclofenac is used to reduce inflammation and as an analgesic in certain circumstances (Choon and Majella 2014). As an anti-inflammatory drug (Samah *et al.,* 2014), it is a strong inhibitor of cyclooxygenase enzyme. Cyclooxygenase enzyme converts arachidonic acid to prostaglandins. There are at least two isoforms of cyclooxygenase: Cox-1 and Cox-2. This drug decreases the production of prostaglandins and thromboxane by inhibiting cyclooxygenase. Accordingly, it shows its anti-inflammation and analgesic impact (Tyler *et al.,* 2014). The aim of our experiment was to provide and introduce a compound that can prevent from creation of pulmonary fibrosis caused by bleomycin prescription or minimize its amount at least. In the previous studies, the treatment approach of this disease was in two general paths: Firstly, using of chemical and vegetable antioxidant compounds to eliminate the created free radicals, and secondly preventing from the inflammation process for the treatment and removal of deposited collagen in the walls of the air sacs. Additionally, studies have demonstrated that non-steroidal anti-inflammatory drugs with specific plant extracts can enhance the analgesic activity. The other its benefit is use it in lower doses and thus limiting its side effects (Marco *et al.,* 2014).

MATERIALS AND METHODS

Bleomycin made in by KAYAKU Company was prepared by injection of ketamine, hydroxyproline, -Klramyn T, Para de Tello amino benzaldehyde, diphenylamine, thiobarbituric acid that all of them have analytical grade of purity. Male Wistar rats, weighing approximately 20 ± 200 g, were kept in the animal house for one week, 12 hours in light and 12 hours in darkness, at temperatures between 20-24°C and relative humidity of 45-55%. After adaptation stage, rats of each group were weighted and marked. All stages of the process were conducted based on instructions of ethics and laboratory animals support committee. Endotracheal bleomycin prescription to create pulmonary fibrosis has the advantage that it creates fibrotic changes in the lung in a short time. For creation of pulmonary fibrosis on the seventh day, before the treatment and after that animals were anesthetized by ketamine (mg/kg 50), 0/30 ml of sterile saline in the negative control groups, and 0/30 ml of sterile saline containing bleomycin in other groups at a rate of one per 100 g of body weight (1IU/Kg) were prescribed for each of the animals. To examined the anti-inflammatory and antioxidant effects of lemon balm, dose of 75 mg / kg and diclofenac 600 µg/kg were selected (Zarei *et al.,* 2014, Javadi and Nasr 1996).

Firstly, the herbal sample of lemon balm (Pharmaceutical Company of Ghol Darou) was prepared. The choice of solvent is the most important factor that should be considered in extracting ingredients of plants. In this study, for the preparation of hydro-alcoholic extract of lemon balm, ethanol solvent 70% was used. To extract lemon balm herb, soaking method was used (Rowe *et al.,* 2014). For this purpose, twigs and dried leaves of lemon balm were powdered by an electric mill, then powder was soaked in hydro-alcoholic mix 70% above the funnel in a way that the level of alcohol level to be above the powder level is a few centimeters. After 72 hours, the content of the container was filtrated with a filter paper and a Buchner funnel. The filtrated solution was transferred into the flask, and its solvent was distillated by a rotary machine and a vacuum pump (set at 40 to 50°C and 70 rotations) until the remaining amount reached to one fifth of the initial amount. The obtained liquid was dried at 37°C. Then, the obtained powder used for the preparation of the hydro-alcoholic in different doses. Groups of case study: In this study, the male rats were randomly divided into three separate groups. The negative control groups received endotracheal dose of 3/0 ml of sterile saline on the first day and in the following days, they were prescribed by solvent of tested drug, sterile saline intraperitoneally. The second group is the group of bleomycin that was prescribed by bleomycin in pre-treatment, sterile saline intraperitoneally. In the eighth day, they received one dose of endotracheal bleomycin of one per 100 g of body weight in 0/3 ml sterile saline and in the other days, they were treated as control group. In the combined group in the first 7 days, 75 mg/kg of lemon balm extract in 3.0 ml of sterile saline, and 600µg/kg diclofenac were simultaneously received intraperitoneally in two separate prescriptions. In eighth day, they received an endotracheal dose of bleomycin of one per 100 g of body weight in 3.0 ml of sterile saline and in the next days this group was treated pre-treatment days. During the experiment, animals must be examined in terms of general and respiratory status, especially until twelfth day when animal is suffering from respiratory problems. The hair and the loss of animal, their daily activities, and their intake food should also be considered. An important factor here is the particular animal weight that until the fifth day, their bodies are weighted daily, then every four or five day they are weighted. At the end of the twenty-first day, by cutting the arteries connected to the lung and other accessories, lung is separated from the chest.

Determination of amount of collagen tissue:

To determine the amount of hydroxyproline, that reflects lung collagen content, a half ml of homogenate fluid is removed and after precipitation of its proteins, its sediments are hydrolyzed for 18 hours at a temperature of 116°C by hydrochloric acid 6 normal.

Its hydroxyproline content is determined according to Woessner (Woessner, 1961). method at a wavelength of 557 nm by a spectrophotometer. The quantification of MDA was done by thiobarbituric acid (TBARS) method through TBA reagent (Take *et al.*, 2012). The absorption of samples was read in the spectrophotometer at a wavelength of 532 and the MDA level was determined. Histology experiments were carried out in two stages of preparation of cut and coloring. Coloring was done by two methods: coloring Hematoxylin eosin and Masson trichrome. For each group of rats, the mean of variable level is expressed as Mean ± SEM and for comparison of means, the analysis of variance (ANOVA) was used. In order to examine and determine the differences of means and the significance of results, Duncan 's test through SPSS software was analyzed. In all tests, P <0.05 was considered as significance level.

RESULTS

Lung morphological studies showed that in the control group, the lungs were totally normal and pink and no edema and blood spots or fibrous tissue were found in tissue and its surface. In the Bleomycin group at the end of experiment, the size of lung was significantly increased and severe edema was visible in the lung. Lung weight in the negative control group, positive control, diclofenac, lemon balm extract and combined group was 1.3±0.04, 2.6± 0.71, 1.6±0.04, 1.5±0.07, and 1.5±0.57, respectively, that was statistically significant compared to control group (P <0.05) (Figure 1). Lung weight ratio to last day weight of the anima in positive control group, negative control and combined group was respectively as 1.09±0.045, 0.57±0.024, and 0.66±0.017, which was statistically significant compared to the positive control group (P <0.05) (Fig. 2). The results showed that the amount of hydroxyproline of bleomycin group has increased by 73% compared to the negative control group that this increase was statistically significant (P <0.05), and in the combined group (lemon balm + diclofenac) the amount of hydroxyproline also increased by 19% compared to the control group that this increase was not significant statistically (P> 0.05). In addition, this factor decreased by 31% compared to bleomycin group that this reduction was statistically significant (P <0.05) (Fig. 3). The amount of malondialdehyde in the lung of bleomycin group of lung increased by 129% compared to the negative control group that this increase was statistically significant (P <0.05) and the amount of malondialdehyde of lung in the combined group (lemon balm + diclofenac) also increased by 7% compared with control group, but this increase was not statistically significant (P> 0.05). This factor reduced by 47% as compared to bleomycin group that this reduction was statistically significant (P <0.05) (Fig. 4). The impact of combination of diclofenac and lemon balm on lung weight (Fig. 1). The ratio of lung weight to weight of last day (Fig. 2).

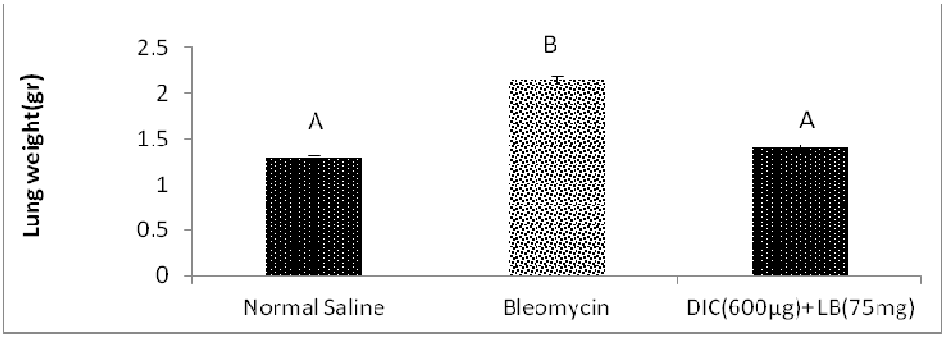

Fig. 1.

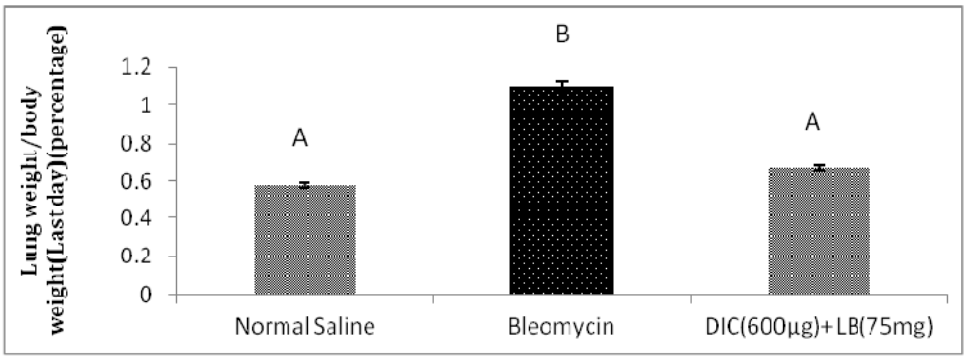

Fig. 2.

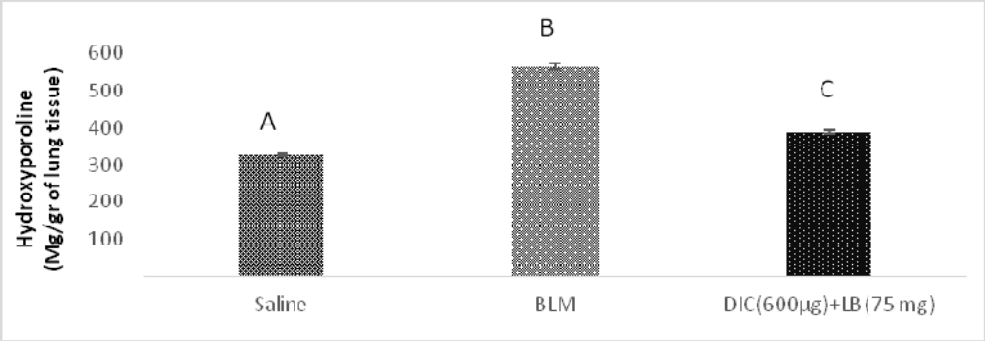

Fig. 3.

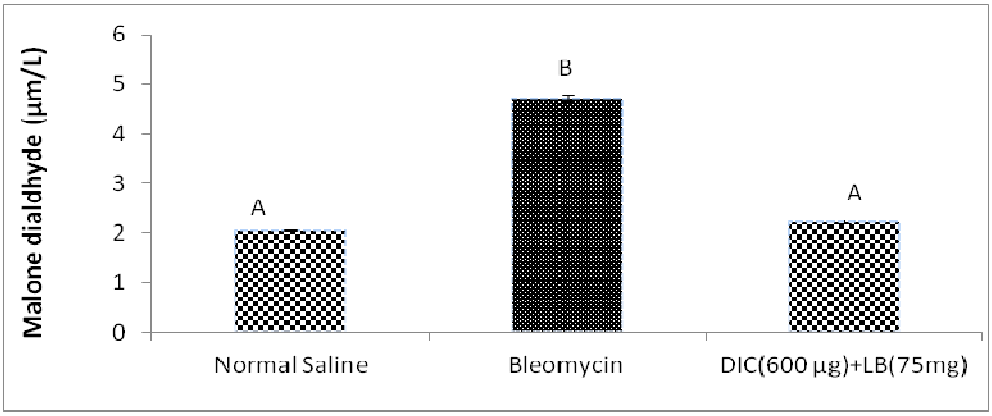

Fig. 4.

The amount of malondialdehyde (Fig. 3), the amount of pulmonary hydroxyproline in the studied groups: using analysis of one-way variance by (One Way ANOVA) and complemantory test of Duncan. Groups with similar colors according to Duncan test ($P < 0.05$) has the same effects but they are not significantly different.

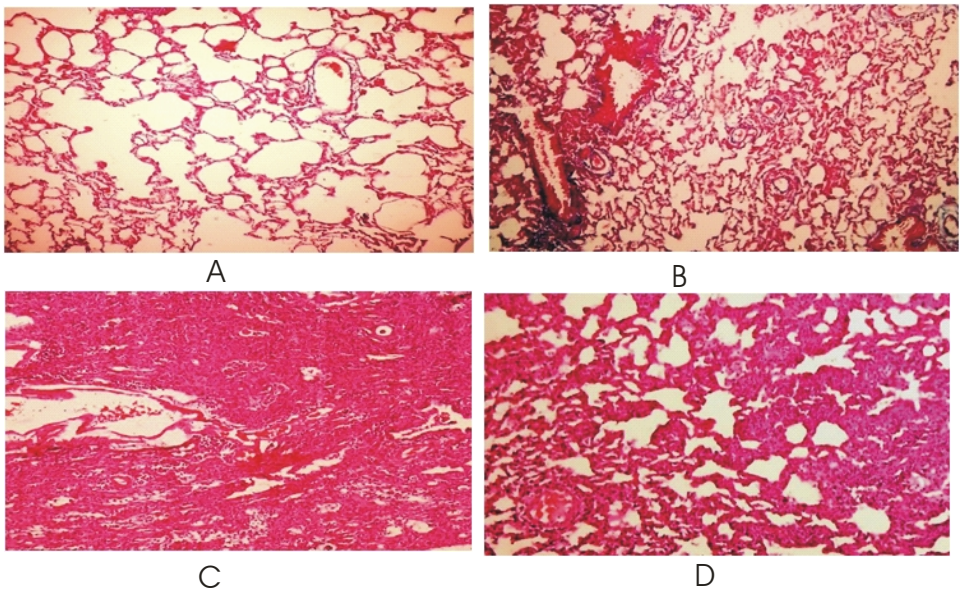

Fig. 5. Results of histology.

Fig. 5. **(**A). Obvious section of lung tissue of rate. Three weeks after the intraperitoneal injection of normal saline, the view of lung tissue was normal and no fibrosis and destruction were found in the lung tissue. Fig. 5. **(**B). Obvious section of lung tissue of rate. In the negative control group that received an intraperitoneal dose of 3.0 ml of sterile saline and during the twenty-one-day experiment they received only sterile saline intraperitoneally. As we can see, collagen deposition around the bronchi is rare and level 1 shows pulmonary fibrosis. Coloring was done by Mason's trichrome method. Fig. 5. (C). Obvious section of lung tissue of rats. It has received two weeks after prescription of a single dose of bleomycin intratracheally. In most parts, the alveolar wall rupture was torn and it has become in the form of open and irregular spaces and level 4 indicates fibrosis. Fig. 5. **(**D). Obvious section of lung tissue of rats. A week before and two weeks after prescription of bleomycin intratracheally, in the combined group (lemon balm extract + diclofenac), most of alveoli were healthy and compared with bleomycin group, fibrosis is seen rarely in interstitial tissue and around the bronchioles.

DISCUSSION AND CONCLUSION

Based on found results, it can be concluded that the combination of these two materials could reduce the rate of progression of pulmonary fibrosis. To create pulmonary fibrosis model, rat was used as experimental animal, since it has been demonstrated that pulmonary toxicity of bleomycin on the lung tissue of rat that received a single dose of bleomycin is similar to pulmonary complications associated with systemic prescription of the drug in humans (Rajasekaran *et al.*, 2013, Liang *et al.*, 2013). Morphological changes showed that bleomycin injection increased the lung weight, which is for two reasons, firstly because of edema and accumulation of collagen fibers, and secondly it caused histological changes such as large-scale destruction of the alveoli, the accumulation of inflammatory cells and accumulation of fibers collagen in the lung tissue in terms of histology. In the event of inflammatory diseases in the lung, parts of the antioxidants produced in the lung cross over cell membranes and they enter into blood stream, leading into oxidation of unsaturated fats. MDA is produced from peroxidation of lipids and its production rate is proportional to the failure and breakdown of unsaturated fatty acids. Therefore, measuring the MDA is an appropriate indicator for lipid peroxidation (Pryor and Stanley) Pathology images related to positive control and treatment groups indicate that factors released by inflammatory cells cause inflammatory reactions in the lungs and eventually cause disruption of the alveolar walls and thickening of them. Therapeutic group with its anti-inflammatory and antioxidant property could prevent the onset or progression of some of these reactions and thus reduce the rate of progression of fibrosis. Pulmonary fibrosis seeks to influence on the extracellular matrix causes collagen deposition and proliferation of cells in the interstitial tissue. A high percentage of these cells include fibroblasts and myofibroblasts, which increase production of pulmonary collagen, leading to pulmonary disability and failure (Davey *et al.*, 2005). Through inhibition of cyclooxygenase 1 and 2 enzymes, diclofenac prevents from conversion of arachidonic acid into interface and the production of prostaglandins. It has been known that bleomycin increases the production of reactive oxygen radicals in biological systems and it can lead into toxicity and side effects, and antioxidants can cut this route (Fazli and Malekirad 2012). The reason of inhibition of collagen deposition due to bleomycin by lemon balm it is not clear, but according to the main phenolic compounds including quercetin and rosmarinic acid, lemon balm has been as a rich source of useful antioxidants and it can increase antioxidant defense activity and decrease triglycerides, cholesterol, and oxidative. Thus, we can conclude that lemon balm acts through taking the active radicals (Dastmalchi *et al.*, 2008). As can be seen, in the combined group, effective results was achieved and better results are even can be achieved through a change in dose and herbal extract.

FUTHER STUDY

This study can contribute to future research on effects of antioxidant and anti-inflammatory properties and reduce the Bleomycin-induced pulmonary fibrosis for helping to Patients with cancer.

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Abbreviations:
LB: Lemon Balm
DIC: Diclofenac
OH-Proline: Hydroxyproline
MDA: Malonedialdehyde

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