Journal of Environmental Treatment Techniques 2019, Special Issue on Environment, Management and Economy, Pages: 874-877

Journal of ENVIRONMENTAL Treatment Techniques

*J. Environ. Treat. Tech.*
*ISSN: 2309-1185*

Journal web link: http://www.jett.dormaj.com

# Comparative Analysis of Biologically Active Substances in Trifolium Pratense and Trifolium Repens Depending on the Growing Conditions

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Received: 05/08/2019 Accepted: 20/11/2019 Published: 20/12/2019

## Abstract

In our studies, we studied the content of pharmaceutically promising biologically active substances, namely phenolic compounds, flavonoids, ascorbic acid, provitamin A (b-carotene) in plants of meadow clover and creeping clover (*Trrifolium repens*). The effect of the plant growth site on the content of the studied compounds was also studied. The obtained data showed that the content of biologically active compounds in white clover (Trifolium repens L.) is similar to those of red clover (Trifolium pratense L.) and white clover is a source of a whole set of pharmacologically active compounds, as well as red clover. The influence of the place of plant growth on the content of the studied compounds was revealed, which may be important for solving production problems. For example, in clover plants of both species studied, the flavonoid content collected in meadow steppes is higher than in other places. Regarding vitamin C, a significant content is shown for plants from floodplain meadows, also for both species. The content of provitamin A and phenolic compounds, according to our data, depended more on the type of clover. So the content of provitamin A was found to be higher in white clover, and phenolic compounds - in red clover.

**Keywords:** Red clover, White clover, Biologically active substances, Favonoids, Isoflavonoids, Phenolic compounds

## 1 Introduction

Throughout the world, interest in herbal medicine is increasing every year, including in our country. The process of growing medicinal plant materials is economically viable, which ultimately has a positive effect on the cost of production and has a slight negative impact on the environment.

The most common plant in our country, characteristic of meadow and pasture biocenoses, is considered clover (Trifolium L.), which genus includes about 65 species (1). In total, this genus has about 300 species. Red meadow Trifolium pratense L. is considered the most studied species, which is an excellent fodder and honey plant, and therefore has widespread use in agriculture (2, 19).

There is a significant amount of data in the literature that the herb Trifolium pratense L. has a whole set of biologically active substances (BAS): triterpenoids, sesquiterpenoids, carotenoids, coumarins, cumestanes, flavonoids, etc. (3). In folk medicine, Trifolium pratense has long been used as a medicine for inflammatory processes in the bladder, for various bleeding, and as a diuretic for edema of cardiac and renal origin (4, 18). These therapeutic properties of clover are due to the content in the aerial part of Trifolium pratense of a number of phenolic compounds, including flavonoids, in particular, quercetin, rutin (3, 5). In addition, isoflavonoids are of particular importance. Also, in the aerial part of red clover, depending on the place of growth, isoflavonoids are represented by biochanin A, genistein, ononin, formononetin, prunetin, daidzein and their derivatives (3, 6). In this regard, Trifolium pratense L. attracts the attention of pharmacologists for experimental studies. Creeping Clover Trifolium repens L., a no less widespread species compared to meadow clover, has not been practically studied, although it can also be of biological value. In addition, it should be borne in mind that the phytochemical composition of plants largely depends on various environmental factors.

The objective of this work is to show the possibility of using white clover (Trrifolium repens L.) as a source of plant material containing biologically active substances.

## 2 Methods

The objects of our research were red clover (Trifolium prätense L.) and white clover (Trifolium repens L.). In order to identify the influence of the plant growth on the content of biologically active substances, plant material was collected in the steppe, which is characterized by water

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deficiency, in a meadow (in the floodplain of the river), where there is plenty of moisture and lighting, and forest edge, where there is also a moisture deficit and lighting. For experiments, the aboveground parts of plants were collected and dried to constant weight.

The quantitative content of ascorbic acid in the aerial part of the studied plants was determined using potassium hexacyanoferrate. Dry plant material weighing 50 mg was triturated using 1.5 ml of 0.1 M citrate buffer (pH 3.69), then transferred to eppendorf tubes and heated in a water bath (20 min at 40°C). After it was centrifuged for 5 min at 12500 g. The supernatant was used for further reactions. The optical density was measured at a wavelength of 680 nm relative to the control solution.

The content of provitamin A was determined by the number of carotenoids isolated from the aerial parts of the studied plants. To obtain an acetone extract, a sample of dry plant material (100 mg) was ground with 2 ml of acetone in a porcelain mortar. The extract was filtered through a paper filter, the extraction was repeated in small portions of the solvent. Then the filtrate was adjusted to 25 ml and used for determination on a spectrophotometer at wavelengths of 662, 644, 440.5 nm. The pigment concentration was calculated using the Rebbelen formula.

To determine the total content of soluble phenolic compounds, they were extracted with water from the dried ground part of plants at the rate of 1.5 ml of distilled water per 50 mg of material for 45 minutes at a temperature of 70°C. The supernatant was centrifuged to separate from the precipitate.

113 µl of Folin-Denis reagent was added to 113 µl of the aqueous extract and stirred, after 3 minutes 180 µl of $NaCHO3$ solution (10%) was poured, mixed, and 1.8 ml of water was added. After 45 minutes, the solution was centrifuged at 16 thousand rpm for 2 minutes. Then, the optical density in the supernatant was determined at a wavelength of 725 nm. For a control solution, 113 µl of solvent was used instead of the extract.

In order to determine the amount of flavonoids in terms of quercetin, the raw materials were crushed to particles with a diameter of 1 mm. Then the raw material was placed in a 50 ml flask weighing 0.2 g, 30 ml of 90% alcohol containing 1% concentrated hydrochloric acid was added. The flask was heated in a boiling water bath for 30 minutes using a reflux condenser. The flask was then cooled to room temperature and filtered through a paper filter into a 50 ml volumetric flask. The extraction was repeated once again in the above manner.

The extracts were filtered through the same filter into the same volumetric flask, the filter was washed with 90% alcohol and the filtrate volume was adjusted with 90% alcohol to the mark (solution A).

For spectrophotometry, 2 ml of solution A was placed in a 10 ml volumetric flask, 1 ml of a 1% solution of aluminum chloride in 95% alcohol was added, and the solution volume was adjusted to the mark with 95% alcohol. After 20 minutes, the optical density of the solution was measured on a spectrophotometer at a wavelength of 430 nm in a cell with a layer thickness of 10 mm. As a comparison solution, a solution consisting of 1 ml of solution A, brought 95% alcohol to the mark in a 10 ml volumetric flask, was used. The content of the total flavonoids in was converted to absolutely dry raw materials and quercetin in percent was calculated by the formula.

Identification of flavonoids was carried out by high performance liquid chromatography (HPLC), which is characterized by high sensitivity and accuracy, allowing to identify the composition of the studied group of biologically active substances. The peaks of compounds detected in the chromatogram were identified using working standards (WS) of quercetin.

## 3 Results and Discussion

Trifolium pratense grows in moderately humid and upland meadows, in forests and forest edges, along river valleys and banks, on mountain meadow slopes, in fields, along field roads. The chemical composition and medicinal properties are studied relatively well. It contains various aromatic compounds, essential oils, fatty acids, steroids, coumarins, flavonoids and many other compounds of pharmacological value.

White clover grows in floodplain meadows, in the steppes, in forests, along river banks, on the side of roads, in wastelands. There is relatively little information about the chemical composition of clover in the literature. Perhaps that is why this type of clover is not popular in pharmacy, unlike red clover.

The vitamin complex of red clover Trifolium pratese is represented by a wide variety of vitamins. There are vitamins with a high content, namely vitamins of group B, C, E, K, as well as provitamin A or β-carotene. The above vitamins are highly important for the human body, especially in childhood. As shown by our studies of provitamin A, it was most found in Trifolium repens, which is consistent with the literature (7,11,15,16) and in all three locations (Fig. 1). It is also important to note that of the three places of growth, plants collected in the steppe meadows have the highest rate.

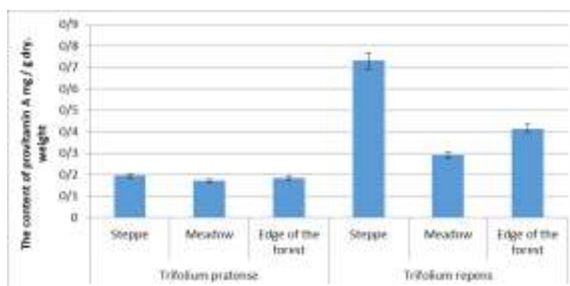


Figure 1: The content of provitamin A in plants *Trifolium pratense* and *Trifolium repens* from different places of growth

Perhaps this is due to the fact that carotenoids perform a protective function, protecting various organic substances, primarily chlorophyll molecules, from destruction in the light during photooxidation, since plants in the steppe zone are more susceptible to direct light rays.

Ascorbic acid has strong antioxidant properties, thereby acting as an important component of the human body's immune system. Together with bioflavonoids, tocopherol and retinol, ascorbic acid acts as a direct-acting bioantioxidant. This complex of compounds with antioxidant properties causes a low level of free radical states of lipids and biopolymers in the cell. Vitamin C and bioflavonoids with simultaneous exposure enhance the effect and complement each other, in connection with which, they often occur together in dosage forms.

The results of the study of vitamin C content were identical for both types of clover (Fig. 2). In this case, the maximum values were found in plants from floodplain meadows. There is evidence that the accumulation of vitamin C in plants is significantly affected by the conditions of their growth. In plants of the northern regions, the content of ascorbic acid is much higher, compared with plants growing in the southern regions. Also, the type of soil on which plants grow affects the content of vitamin C. It has been shown that on heavy soils the synthesis of ascorbic acid in plants is weaker than on light soils (8, 13).

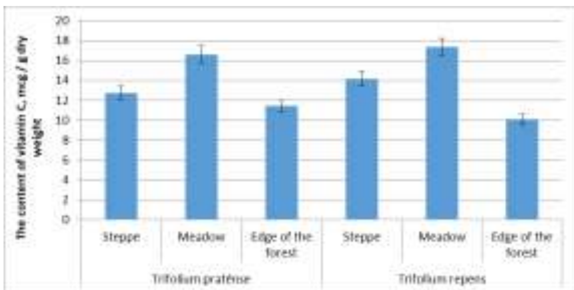


Figure 2: The content of vitamin C in plants *Trifolium pratense* and *Trifolium repens* from different places of growth.

Phenolic compounds of plants are usually the products of secondary metabolism, which are represented by a large group differing in structure, chemical properties and biological activity. Phenols in plants are found in all organs, especially a significant concentration is found in actively functioning organs. The content and concentration of phenolic compounds also depends on the type of plant. Plants of the same genus can differ greatly in the content and concentration of phenolic substances and in their qualitative composition.

Plants of Trifolium pratens compared to Trifolium repens showed a higher total content of soluble phenolic compounds (Fig. 3). Apparently, this indicator depends on the type of plants, as in the case of provitamin A, since the results are higher in all three locations.

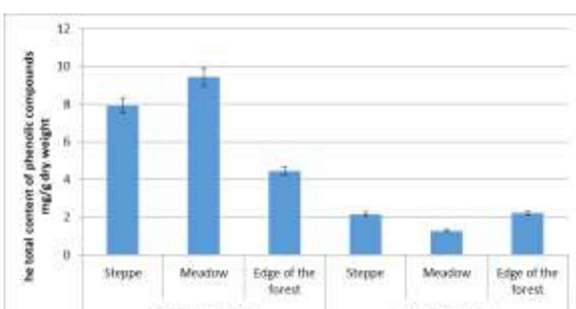


Figure 3: The content of phenolic compounds in plants *Trifolium pratense* and *Trifolium repens* from different places of growth.

The maximum value of the studied parameter for meadow clover was found in raw materials from a floodplain meadow, and the minimum value was found from the forest edge, which is opposite to the data obtained for creeping clover. Possibly, these results can be explained by the fact that most of the secondary metabolites are represented by phenolic compounds, which are formed in large quantities when plants are exposed to stress factors.

The phenolic compounds contained in red clover are mostly represented by flavonoids. The chemical structure of flavonoids presents a phenylpropane skeleton with the formula C6-C3-C6. These compounds are interesting in that they exhibit diverse biological activity (10, 12). This is probably due to the presence of an oxygen atom in the ring of these compounds.

Given the wide range of pharmacological effects of flavonoids, namely immunomodulatory, antispasmodic, anti-stress, capillary-strengthening, anti-toxic, anti-atherosclerotic, anti-carcinogenic, etc., the value of Trifolium pratense as a medicinal plant becomes clear. Among the huge variety of Trifolium prenens flavonoids, some of them can be noted: flavones (apigenin, baicalein tricetin, and luteolin), isoflavones (biohanin-A-7-glucoside, prinetin, formononetin, and ononin), and flavonols (rutin, kempferol, isoquercetin, etc.) (14).

Our data on the study of flavonoid content depending on the place of growth were approximately similar for each of the studied clover species (Fig. 4). The highest values were obtained from the raw materials of meadow steppes, and the lowest - from the edges of the forest. If neglecting the significance of differences between the variants, for the studied compound, namely for flavonoids, white clover is not much inferior to red clover.

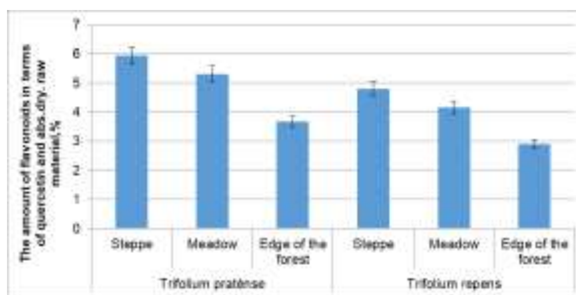


Figure 4: The content of flavonoids in terms of quercetin in plants *Trifolium pratense* and *Trifolium repens* from different places of growth.

## 4 Summary

The obtained data showed that the content of biologically active compounds in white clover (Trifolium repens L.) is similar to those of red clover (Trifolium pratense L.). The influence of the place of plant growth on the content of the studied compounds was revealed, which may be important for solving production problems. For example, in clover plants of both species studied, the flavonoid content collected in meadow steppes is higher than in other places. Regarding vitamin C, a significant content is shown for plants from floodplain meadows, also for both species. The content of provitamin A and phenolic compounds, according to our data, depended more on the type of clover. So the content of provitamin A was found to be higher in white clover, and phenolic compounds - in red clover.

## 5 Conclusions

The results of analysis of the obtained data of the study of the phytochemical composition of two types of clover show that white clover is a potential source of a number of pharmacologically active compounds, like red clover, which creates the basis for a deeper, more detailed study of it as a possible object of phytotherapy methods and treatment directions, since the species under study refers to the sources of natural herbal preparations.

## Acknowledgments

The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University, as well as with the financial support of the Russian Foundation for Basic Research and the Government of the Republic of Tatarstan in the framework of the research project No. 18-44-160015.

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