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Research Article

Raw material "Trifolii pratense herba" originated from southern Ukraine: diagnostic microscopic features and its antioxidant activity

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Abstract

As a result of pharmacognostic and pharmacological studies, diagnostic signs of *Trifolii pratense* grass collected in the phenophase of active flowering in the area of southern Ukraine were revealed. We have proposed the adoption of an appendix to the existing monograph of the State Service of Ukraine on Medicines and Drugs Control "Trifolii inflorescences", with raw material of grass *Trifolii pratense*, with appropriate microscopic identification of diagnostic signs of leaves and stems of the plant. According to our previously found optimal conditions for extraction of medicinal plant raw materials (ratio of solvent-plant material, time and temperature of extraction), selected aqueous extract (1: 5) of clover grass with yields of maximum active substances to the extract from 60 min to 24 hours, which showed lower values of TBARS (0.541 ± 0.0291 μM / g, mean ± SD), which gives grounds to recommend the use of raw materials as an antioxidant, compared with the action of ascorbic acid.

Keywords

antioxidant activity, microscopic diagnosis, phytochemistry

Introduction

Legumes (Fabaceae L. or Leguminosae L.) are one of the largest families of flowering plants, comprising three subfamilies, namely: Caesalpinioideae, Mimosoideae and Papilionoideae (Rendle 1952; Willis 1966; Takhtajan 1969). The family has 600–700 genera and more than 12,000–17,000 annual and perennial species of grasses, shrubs, vines, rarely trees (mostly in the tropics). Ukraine is home to more than 60 genera and 1,800 species of this family (Takhtajan 1969).

The genus of clover named *Trifolium* L. is one of the tallest angiosperms and perennial herbaceous plants of the Leguminosae L. family and includes a large number of species distributed throughout the European continent (Taia 2004; Fawzi 2011). In the south of Ukraine *T. pratense* prefers to grow floodplain meadows, steppe slopes and edges.

We have found carefully described recommendations for the identification of a typical sample of *Trifolium pratense* in nature and among herbarium specimens. However, a large number of intermediate (transitional) forms grow

around, combining features of *T. pratense* (*T. repens*, *T. incarnatum* and other species of *Trifolium*), which seriously complicates the bookmarking of herbarium specimens and their identification in nature. In addition, the botanical description of the morphology and anatomy of the plant does not coincide with the pharmacognostic diagnostic features in the commodity analysis of medicinal plant raw materials. The task of this analysis is to find among all structures the certain specific and diagnostic features (Rudall 2007; Fawzi 2011; Zoric et al. 2012; Ghosh 2018).

Previous researchers have described the raw material *Trifolium pratense* as an inflorescence that is cut along with the apical leaves during full flowering of plants (Booth et al. 2006; Tuttolomondo et al. 2014). From previous sources it is already known about the research of flowers, or the specification of the type of medicinal plant raw materials was not specified at all. The State Pharmacopoeia of Ukraine contains the monograph "Inflorescences of Trifolii pratense" with a diagnostic pharmacognostic description of raw materials - flowers.

However, some literature sources consider clover as a whole plant (grass), based on a more careful attitude to this biological resource and given the relatively low ability to regrow after pruning.

Thus, we are faced with the expediency of conducting microscopic diagnostic pharmacognostic analysis of the herb *Trifolium pratense* L. as a source of pharmacopoeial plant raw materials growing in the south of Ukraine. We considered it expedient to conduct research and find chemotaxonomic markers based on the similarities and differences in the anatomical structure of the flora of the south of European continent (Zoric et al. 2012; Ghosh 2018). The ability to reduce the formation of free radicals, their absorption or uptake of reactive oxygen species (ROS), described by previous researchers and is an asset of great interest in natural compounds of T. pratense (Burmaka et al. 2010; Ertas et al. 2015; Zhang et al. 2020).

We found reports on the prevention or reduction of degenerative diseases such as cardiovascular and cancer. Compresses and irrigation are used for abscesses and thermal impressions, for joint pain; fresh crushed leaves are applied to purulent wounds and ulcers; in folk medicine, fresh plant juice is used to treat allergic eye lesions (Tuttolomondo et al. 2014; Wagay 2014). Indian Red Clover has been described as a medicine for burns, bronchitis, and a sedating medication (Wagay 2014). Have been studied raw materials (leaves) of *Trifolium pratense* and *T. pratense* subsp. *nivale*, grown in Italy by Italian phytochemistry sciences (Tava et al. 2019).

The researches of scientists show the accumulation in plants of the genus *Trifolium* L. not only a large number of proteins with a certain energy value, but also biologically active secondary metabolites of natural origin.

At present, there is an active phytochemical studed of members of the genus Clover. We know about high amounts of glycosyl and glycosyl malonate derivative of flavones (quercetin in leaves and quercetin and kaempferol in flowers), isoflavones (biochanin A and formononetin in leaves and flowers were detected in red clover (Taia 2004; Dabkevičienė et al. 2012; Zoric et al. 2012; Zhang et al. 2020). Known that have been identified and quantitatively described pratensein, macaine, medicarpine, irilon, dihydrobiochanin A, cicerin, quercetin, kaempferol, coumestrol in *Trifolium pratense* L. (Tava et al. 2015); daidzein and genistein (Dabkevičienė et al. 2012) and isoramnethin (Burmaka and Kolyadich 2010) in *Trifolium* species and their aerial plant parts). Phytochemical sciences have been noted the use of *Trifolium pratense* L. in polycystic ovary syndrome and estrogenic medicines by Red Clover flavonoids and their glycosides prunethin-4' -O-a-D-glucopyranoside, genistein-7-0-p-0-galactopyranoside, prunethine-4'-O-p-O-glucopyranoside (Goswami et al. 2012; Spagnuolo et al. 2014). T. Muzashvili et al. studied 88 Clover species for the content of cyanogenic glycosides (Muzashvili et al. 2014). A wide range of chemical structures in the raw materials of clover species is the key to excellent antioxidant activity (Dabkevičienė et al. 2012; Tuttolomondo et al. 2014).

From previous studies, malonic dialdehyde (MDA) is known as one of the important oxidation products in lipid peroxidation (Al-Rimawi 2015). MDA reacts with thiobarbituric acid (TBA) to form a colored compound that can be determined chromatographically, spectrophotometrically, and by other methods. Usually TBA reacts with several reactive capable substances in biological samples. Therefore, modified terminology is widely used today, such as: reactive substances of thiobarbituric acid (TBARS).

From previous studies, several methods are known to assess the level of TBARS using high performance liquid chromatography (HPLC), which are characterized by high cost, long operation time of chromatographic equipment and highly qualified specialists. Scientific search for simpler methods is an important area. Zeb and Ulla (2016) in their publication reported on the development of a spectrophotometric method of MDA analysis in fried foods such as cookies, chips and other fast food samples. The method of these authors can be compared with the results obtained by HPLC.

Thus, the second task of our study was to determine the antioxidant capacity of the aboveground part (grass) of clover meadow area of southern Ukraine by BARS level and analysis of these indicators (statistically, Pearson's correlation coefficient and clarity of the box diagram).

Materials and methods

Reagents

Crystalline chloride, crystalline thiobarbituric acid (TBA) 99.5–101% of pharmacopoeial purity in the original packaging produced by Sigma, Germany were used for research. Trichloroacetic acid with a purity of 98% (Erba Lachema, Germany) was used. Malonaldehyde bis (dimethyl acetate) - purity for analysis of Sigma-Aldrich production, Germany. 98% crystalline sodium hydroxide, chemically pure produced by Brenntag CEE Austria. N-butanol for analysis was used 99.9% pure for analysis produced by Merck, Germany.

Collection of plant materials

Plants Trifolium pratense L, which were prepared for research, were grown from the collection seeds of the Department of Botany of the Botany Department of Karazin National University, Kharkiv, Ukraine. Swards (grass) were collected in the suburbs of Zaporizhzhia (urban settlement Primorske [47°37'28"N, 35°17'39"E] during the 2019–2021 seasons. Plants were cultivated under the same conditions and harvested in the same period to exclude the dependence of changes in the structure of raw materials and leaf epidermis on external living conditions, seasonal weather conditions and age in the phenological period of full flowering (May-June). Pre-dried raw materials were dried under cover or in a well-ventilated place, making sure that the inflorescence did not dry out and crumble. We received 20% of dry raw materials.

The identification of the species was confirmed at the Botany Department of V. N. Karazin Kharkiv National University, Ukraine (Head of the Department, Herbarium Keeper, Assoc. Prof. Gamula Yu.G.).

Microscopic studying

For microscopic analysis used temporary preparations of leaves, stems and flowers, which were pre-boiled in a solution of sodium hydroxide (5%, aq.) without the use of dyes; cross-sections were made with a blade (Grechana et al. 2020). On temporary preparations we determined the shape of epidermal cells, type of respiratory system, vascular tissue, structure of trichomes, glandular (secretory) trichomes and more. Studies of the drugs were performed on a microscope "Axioskop 40" (Zeiss, Germany) at a magnification of 15 × 40. Photographs were taken with a Canon PS G7 (Japan), measurements were performed in 20 replicates using the licensed program AxioVision Rel. 4.7, statistical processing (average value, error, coefficient of variation) was performed using Microsoft Excel. Anatomical parameters of the epidermis were considered low-variable if the coefficient of variation of Cv is less than 20%, as moderately variable - at Cv > 20%, and highly variable - at Cv > 40% (Zakharevich 1964). The type of stomata was determined according to the classification of Baranova (1985), the description of epidermal cells was performed according to the method of SF Zakharevich (1964); Butnik and Timchenko (1987), the respiratory index was calculated by the formula of A. Kästner (Kästner 1972).

Antioxidant activity

Determination of antioxidant activity was performed according to the readily available protocol for non-enzymatic lipid peroxidation (Kohn and Liversedge 1944; Guichardant et al. 2011) free radical (iron ion, II), described by Bayir et al. (2020) and Durojaiye and Adewale (2013). The results were stated using a spectrophotometer.

The grass of the plants was placed in the oven of the universal Memmert UF55 (Memmert, Germany) at 80 °C for 24 hours. The dried plant raw materials were ground to a powdery state with a pestle and mortar. Samples (100 mg) were extracted by extraction with aqueous-ethanol mixture (70%, 10.0 ml) and distilled water (5.0 ml and 10.0 ml), using a Soxhlet-type extractor Behr R 304 (Germany). Our previous studies have tested the optimal residence time of the mixture in the extractor with the highest yield of components to the extractant - 60 minutes - 24 hours. Based on this, we performed extraction during the day without heating - at room temperature 20 ± 2–3 °C. Three batches were prepared for each extractant. Accordingly, three extracts were obtained (alcohol, water 1:5 and water 1:10). The extracts were dried using a Freeze Dryer FD-10 (China). Stored at a temperature of 4 OS until the study. Immediately before measuring the level of TBARS, the extracts were diluted by adding 2 ml of distilled water.

As a substrate was used a suspension of egg lipoproteins (homogenized chicken egg yolk with 1 liter of phosphate buffer, pH = 7.4).

Two ml of tested extracts were added to a suspension of egg lipoproteins and 10% aqueous $FeSO_4 \times 7H_2O$, mixed thoroughly and incubated with a Memmert UF55 oven (Memmert, Germany) for 60 min at 37 °C. To stop the reaction and prevent further oxidation of the medium, 2 ml of a 20% mixture of trichloroacetic acid with Trilon B was used. The samples were shaken for 1 h with a Wisebath bath shaker WSB-18-1257-00010 (DAIHAN Scientific, South Korean) and filtered laboratory deashed blue tape (LLC "VDP Aquachim", Ukraine) using laboratory small recirculating pump water SHZ-DIII (Biobase, China). The filtrate was centrifuged for 30 min on a SM-6M centrifuge (ELMI SIA, Latvia), the supernatant was drained and used for research.

The supernatant (1 ml) was added to a 10 ml tube and mixed with 1 ml of 0.8% aqueous thiobarbituric acid (TBA). TBA solution, according to Ohkawa et al. (1979) and Wang et al. (2018), was prepare daily fresh.

The mixture was heated on a boiling (95 °C) water thermostatic bath VB-20 (MICROmed, Ukraine) for 60 minutes. The tubes were rapidly cooled to room temperature under running water. The formation of a pink chromogenic trimethyl complex was observed. The stained complex of TBA-active products (TBARS) was extracted with n-butanol. The concentration of TBARS was determined spectrophotometrically on a digital spectrophotometer UV-VIS PD-30000UV (Apel, Japan) in comparison with n-butanol at wavelength (λ = 532 nm). Determination of indicators for each sample was repeated (n = 6) according to the above procedure.

We selected the known antioxidant ascorbic acid (gamma-lactone 2,3-dehydro-L-gulonic acid, solution for injection, 50 mg / ml, 2 ml, PF Darnitsa, Ukraine). With two ml of ascorbic acid solution instead of plant extract did the same steps as described above with repeating the procedure, n = 6.

A "zero" study was performed, where instead of extracts 2 ml of distilled water were taken and in the repeatability n = 6 and all the above experimental actions were performed.

The calculation of antioxidant activity (AOA,%) was performed according to the formula 1:

$$AOA = \frac{(E_c - E_e)}{E_c} \times 100\ \% \quad (1)$$

where: E c - optical density of the test extract, E e - optical density of the extractant TBARS (n-butanol).

Data were processed using statistical methods and evaluated both parametrically (by Student's coefficient, t-test) and non-parametrically (by Mann-Whitney test - u-test). We presented a comparative evaluation of the data obtained using Pearson's pairwise correlations and scale diagrams described by Vehkalahti and Everitt (2018).

Results and discussions

Microscopic analysis

The anatomical structure of the flower, the leaves of bract, petals and generative organs has been described in the literature (Pinar et al. 2001; Halbritter and Auer 2021) and in the State Pharmacopoeia of Ukraine (2019). Therefore, we did not focus on the widely described elements of the studied plant.

The leaves are trifoliate, tough. Leaflets have 2–3 cm in length and 1–1,5 cm in width, in obovate shape, pointed at the end, finely or unevenly dentate along the edges with a dense network of lateral veins that are thickened towards the edges.

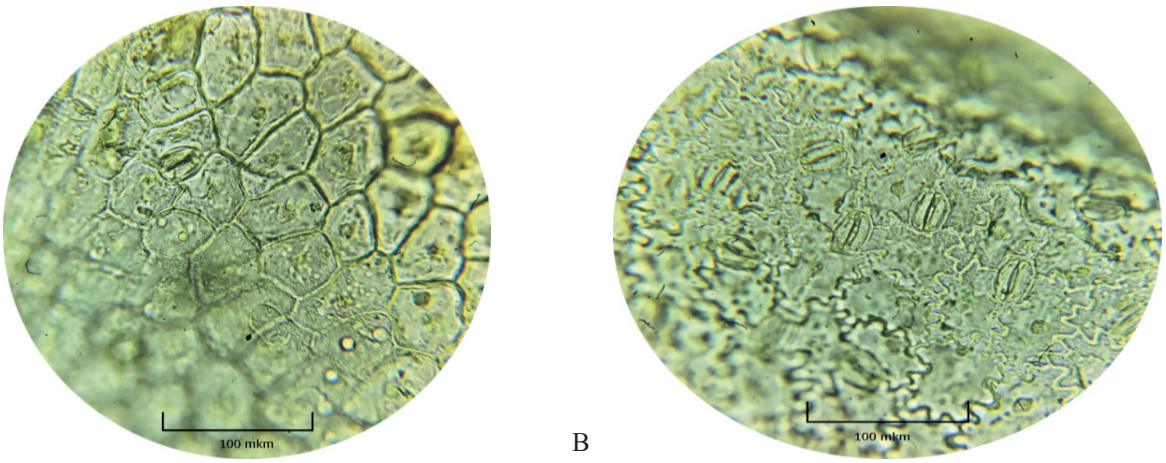
The leaf is amphistomatous (stomata are present on the upper and lower leaf surfaces). Among the epidermal cells are pairs of sausage-shaped guard cells (pore or stoma). The morphometric characteristics of the leaf epidermal structures are given in Table 1.

Leaf adaxial epidermis (Fig. 1A) The main epidermal cells are polygonal or irregular, with rectilinear-rounded outlines, from 51.70 to 84.77 microns in length and from 38.64 to 61.97 microns in width. The anticlinal walls are arcuate or wavy. Epidermal cell projection is polygonal, rounded or flattened, compactly arranged. The major epidermal cells are thick-walled, their number varies from 347 to 425 per 1 mm^2. Intercellular spaces are absent. Adjacent boundaries angles are obtuse, rounded, pointed, and straight.

The stomata are solitary, chaotically arranged. The stomata apparatus have anomocytic type. Stomata have surrounded by 3–5, more often 4 cells. Peri-stomata cells that neighbor with guard cells do not differ in shape and size from the main epidermal cells. A definite orientation of the peri-stomata cells has not been observed, however, in some cases, ones are located in a certain way: two cells are border with the guard cells lateral sides (parallel into stomata long axis) and two

Table 1. Morphometric characteristics of *T. pratense* leaf epidermal structures.

Leaf side		Upper epidermis	Lower epidermis
Leaf type		Amphistomatous	
The character of the AW ELC		Straight-line, rectilinear-rounded	Sinuous
The character of the ELC area projection		Polygonal, flattened	Flattened
ELC average number, pieces per mm^2		404.38 ± 22.55	436.18 ± 16.20
Cv, %		13.76	15.41
ELC average size, mkm	Longitude	63.82 ± 2.59	57.11 ± 2.30
	Cv, %	15.83	18.41
	Width	35.41 ± 1.52	30.14 ± 1.60
	Cv, %	16.8	21.13
ELC average area, mm^2		0.002369 ± 0.00039	0.002017 ± 0.00016
SA type		Anomocytic	Anomocytic
Stomata average number, pieces per mm^2		32.40 ± 3.88	94.82 ± 10.2
Cv, %		20.46	24.15
SI, %		7.42	17.87



A B

Figure 1. *T. pratense* leaf epidermis. **A**. upper epidermis; **B.** lower epidermis

- with the stomata poles. Nevertheless, since the peri-stomata cells do not differ in structure, shape, and size from the rest of epidermis cells, in this case, we also referred to this type of stomatal apparatus as the anomocytic one. The stomata are elongated-rounded shape, their number per mm^2 varies from 25 to 43 pcs. The stomata index is 7.42%. The upper epidermis has no pubescence.

Leaf abaxial epidermis (Fig. 1B, Table 1) The major epidermal cells are irregular in shape with irregular coarsely wavy anticlinal walls and coarsely tortuous outlines, light green, covered with thin cuticle and is interrupted by stomata. Intercellular spaces are absent. The wave's frequency and amplitude are not constant. The sizes of epidermal cells vary over a very wide range from 39.79 to 80.34 microns in length and 21.46 to 45.55 microns in width. The epidermal cells are thick-walled, in various number (from 329 to 511 per 1 mm^2), with their plan projection is stretched out or spread out. Angles are obtuse, pointed, rounded in adjacent boundaries.

The stomata are solitary, in chaotic locations. The stomata apparatus is an anomocytic type: the stomata are surrounded by 3–5, more often 4, cells. As at the upper epidermis, peri-stomata cells that guard cells adjacent, do not differ from the main epidermal cells along with shape and size. Peri-stomata cells' certain orientation is not noted, however, some cases are the same and have characterized to upper epidermis too. The stomata are rounded, in number per 1 mm^2 varies from 67 to 183. Stomata index 17.87%. There are single-row, unicellular simple long hairs with a warty cuticle. The trichomes are formed by cuboid cells with an elongated apical cell. There is a base hair site in a rounded shape, which is surrounded by 12 polygonal epidermal cells, diverging in radial directions (Fig. 2).

PS: AW - anticlinal walls of epidermal cells; ELC - epidermal leaf cells; SA - stomatal apparatus; SI - stomatal index; Cv - variations coefficient.

The central vein on a cross-section has a rounded shape (Fig. 3A). Epidermis cells above the vein are parenchymal. Veins represent vascular bundles. They are founded regularly in the mesophyll. The largest and the oldest vein is founded in the center (midrib vein).

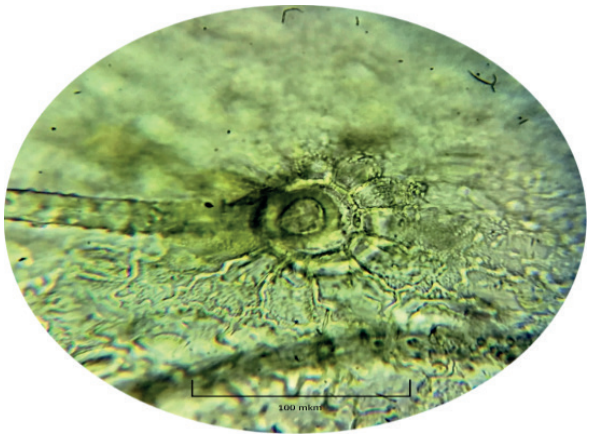


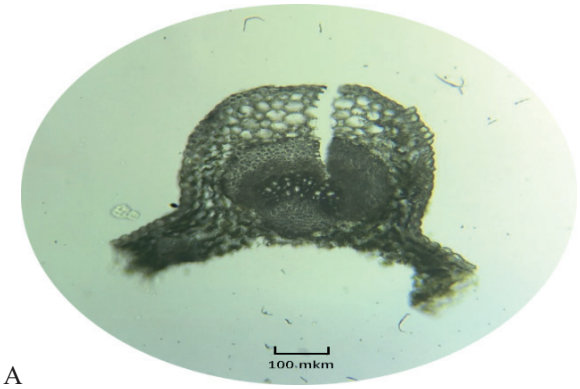
Figure 2. *T. pratense L.* leaf epidermis trichomes

Each vein has a bundle sheath composed of a single layer of compactly arranged barrel-shaped parenchyma cells. The bundle sheath encloses both xylem and phloem. In the xylem, many protoxylem and metaxylem vessels are found. Protoxylem orients towards the upper epidermis. Hence, the vascular bundles are described as conjoint and collateral with the end- arch xylem. The bundle sheath of the midrib vein is connected to the upper and the lower epidermal layers by many layers of collenchyma cells, representing bundle sheath extensions or hypodermal collenchymas. A characteristic feature of the central leaf vein is the crystalline coating, which is formed by single calcium oxalate crystals (Fig. 3B). The vascular bundle is covered by a bundle sheath of parenchyma cells. Fibers are absent in both xylem and phloem. The xylem and phloem elements are conspicuous only in large vascular bundles.

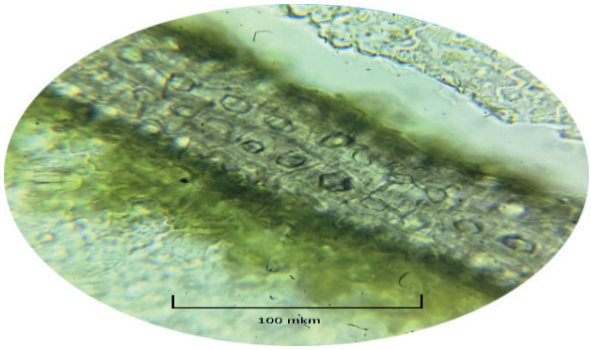
The stem is rounded with slightly protruding ribs. Central axial cylinder is fascicular type: the fascicules are open and collateral, arranged in a circle. There is a sclerenchyma lining in the phloem side fascicules (Fig. 4).

Epidermal cells are elongated parenchymal with thickened rectangular walls, permeated by straight pores; without any pubescence (Fig. 5A). There is a two-layered lamellar collenchyma under the stem rib epidermis (Fig. 5B).

Antioxidant activity: Studies of the antioxidant activity of meadow clover grass were performed with plant excerpts (extracts) available for production in the pharmacy network



A



B

Figure 3. *T. pratense* leaf central vein (cross section, **A**); crystal vein leaf coating (**B**)

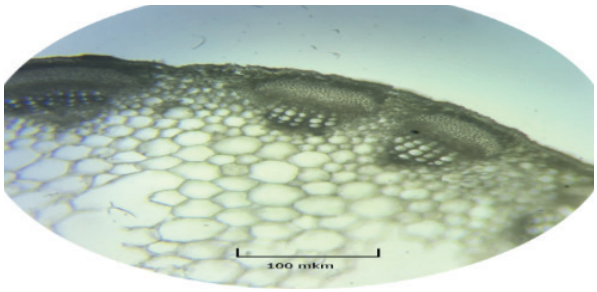


Figure 4. *T. pratense* stalk (cross section)

and everyday life of ordinary consumers (ethanol and water). Previously, we tested the optimal terms, the temperature of obtaining the highest content of active substances of grass extracts of the studied plant, and we studied different ratios of plant raw materials: water (1: 5 and 1:10). Brief descriptive statistics of the experimental results are shown in Table 2.

The aqueous extract of clover grass (1: 5) had the lowest TBARS values among the three subjects under investigation (respectively: 0.541 ± 0.0291 μM / g; 0.638 ± 0.0218 μM / g and 0.570 ± 0.0341 μM / g, mean ± SD).

Table 2. Descriptive statistics of thiobarbituric acid reactive substances (TBARS) in samples *T. pratense* extracts.

	Water 1:5	Water 1:10	Ethanol	Ascorbic acid	Control gr.
mean	0.540967	0.637733	0.569633	0.436517	0.733817
std	0.071296	0.053348	0.083636	0.015618	0.059056
mad	0.057922	0.039867	0.063911	0.010589	0.045811
skew	0.934693	0.502834	0.632727	2.073499	-0.26601
kurt	-0.8193	0.575	0.211965	4.468151	-1.79543
sem	0.029107	0.021779	0.034144	0.006376	0.024109
m	0.00713	0.005335	0.008364	0.001562	0.005906
Me	0.5113	0.63875	0.5441	0.43065	0.74025
M ± sem	0.541 ± 0.0291	0.638 ± 0.0218	0.570 ± 0.0341	0.437 ± 0.0064	0.734± 0.0241
% to mean contr	-26.28	-13.09	-22.37	-40.51	0
contr gr t-test	$p < 0.001$	$p < 0.05$	$p < 0.01$	$p < 0.001$	$p = 1$
contr gr u-test	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p > 0.05$

According to all studies, the degree of belief in the results was within normal limits: from $p < 0.001$ (t-test in aqueous extract 1: 5 and a solution of the reference substance of ascorbic acid) to $p < 0.05$ (t-test in aqueous extract 1:10).

The mean and median values in the tested extracts exceeded the modal values, which characterized the obtained data with a positive skew (skew was in the range from 0.5 to 1). The value of skew for ascorbic acid solution (exceeded 1) characterized the presence of large data skew.

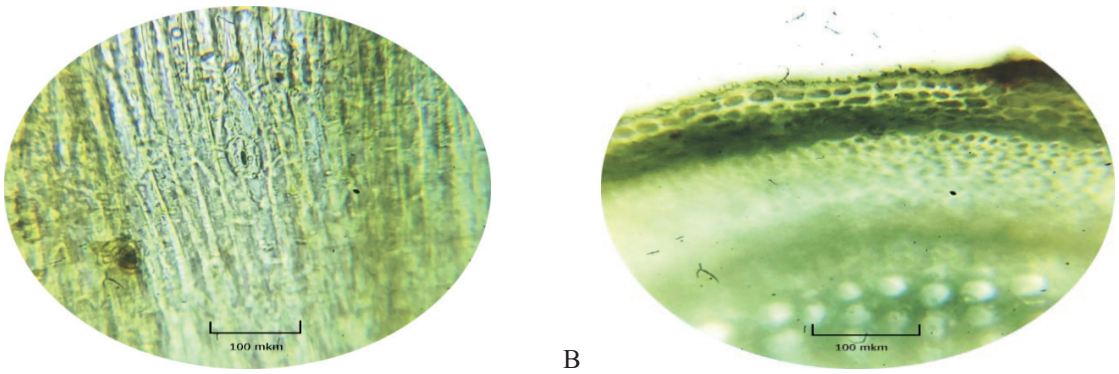
The level of excess, calculated for the studied extracts, indicated the absence of miscarriages in the data set (kurt = -0.8193; 0.575; 0.211965, respectively). An excess of more than 3 in ascorbic acid indicated a miscarriage in the dataset (kurt = 4.468151).

In the case of the "zero" study, where the equivalent volume of water was analyzed instead of extracts, the antioxidant activity index had the highest level (0.734 ± 0.0241 μM / g, mean ± SD). But the degree of belief in the values obtained in the "zero" study does not allow to take into account these figures (t-test $p = 1$; u-test $p > 0.05$).

According to descriptive statistics, TBARS was most pronounced (reduced effect of active products) when using aqueous extract (1: 5). Here, the indicator% to mean contr was equal to -26.28% ($p < 0.001$ *, $p < 0.01$ **) under the influence of the reference drug -40.51 ($p < 0.001$ *, $p < 0.01$ **) relative to the control group.

Us the measure of correlation we were used in this study classical Pearson correlation, which represents the linear relation between two variables. Pearson correlation was computed using the stats R package (Table 3). Overall, there were moderately positive and negative pairwise correlations between scavenging assays, ranging from $r = 0.76$, $P < 0.01$ (water extract 1:5 vs. water extract 1:10) to $r = -0.70$, $P < 0.01$ (water extract 1:5 vs. ethanol extract).

We have been built boxplots that provide a useful way to visualize the range and other characteristics of responses for data groups and to find about the optimal solvent and its ratio with plant material in an expression of maximal antioxidant action (Fig. 6). The boxplot shows that the difference between the medians of the two groups is approximately 1, we can conclude, with 95% confidence, that the true medians do differ. Our investigation had not any data points beyond the whiskers (no outliers), so the whiskers extend to the most extreme data points. The center of distribution water 1:5 is the lowest of the three distributions (median is 0.5113). Q1 was for it far minimum than all extracting data. Moreover, the line marking the median crosses the boxes to-



A B

Figure 5. **A**. *T. pratense* stem epidermis; **B**. *Trifolium pratense* stem lamellar collenchyma

Table 3. Pairwise Pearson correlation of thiobarbituric acid reactive substances (TBARS) in samples *T. pratense* extracts, n = 6.

	Water 1:5	Water 1:10	Ethanol	Ascorbic acid	Control group
Water 1:5		0.76	-0.7	-0.13	0.51
Water 1:10			-0.53	-0.5	-0.11
Ethanol				-0.35	-0.32
Ascorbic acid					0.2
Control group					

ward Q1 indicating a distribution with asymmetrical shape (positively skewed) in this distribution. Distribution water 1:10 is the most concentrated, with mine interquartile range of the three distributions while with the highest center of distribution of the three distributions (median is 0.63875). The centers of distribution in the boxes varied, showing asymmetric shifts, where the smallest with a value of 0.55 was observed in the aqueous extract (1:5) of clover. The data of ethanol extract have the longest asymmetric fences.

Thus, based on the positive correlation coefficient and Fig. 6, we concluded the possibility of further recommendations for in-depth pharmacological studies of the phytocomposition *Trifolium pratense* herb (water_1: 5) as an antioxidant.

to call attention to the advantages of the application of boxplots in data analysis of pharmacognostical interest and, most important, can be very useful to uncover information hidden.

Conclusion

According to the results of this study, we distinguished and diagnosed the main microscopic features of clover grass, which is important in confirming the identity of raw materials in the pharmacognostic analysis, as required by the State Pharmacopoeia of Ukraine.

We proposed the identification of grass-clover by the following microscopic diagnostic features: the cells of the upper epidermis of the leaf are quadrangular rectangular, the lower - tortuous, parenchymal with slightly thickened shells. Anocytic-type stomata, sparse on the upper and very frequent on the lower epidermis, surrounded by 3, rarely 4, periapical cells. The pubescence is characteristic of the lower epidermis - unicellular hairs with a warty cuticle have a rosette of cells at the site of attachment of the hair to the epidermis. A characteristic feature of the central vein of the round leaf is

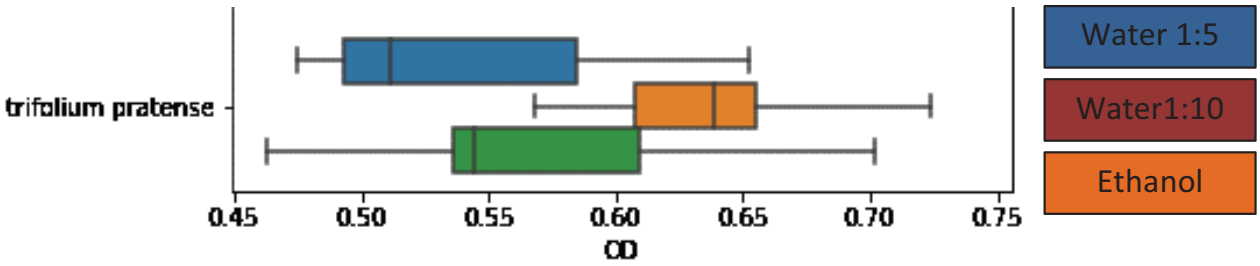


Figure 6. Schematic boxplots for the influence of extractant and its quantity on antioxidant action of *T. pratense* phytocompositions. Units are on the Pauling scale.

Describe *T. pratense* extract inhibited the growth and the proliferation of cancerous cells but its use in clinical setting is limited due to the therapeutic and toxicity doses. Today, complementary compounds can be used to reduce the side effects and enhance the anticancer potential of chemotherapeutic drugs. Therefore, those signs in the anatomical structure of the plant are important for the correct identification and belonging of the plant.

Nevertheless, with such a wide phytochemical and pharmacological interest in studying a large number of clover species, we found few articles about anatomical microscopic identification of Trifolii pratense herbs as medicine (Booth et al. 2006; Zoric et al. 2012) and about coumarins, especially simple coumarins, with anticoagulant action because plants can be regarded as "living factories" producing a variety of chemical compounds, including primary important metabolites for the plants' growth and secondary metabolites. All these components may work together to deliver a synergistic effect in the finished product. Finally, the author team want

the presence of a crystalline coating of single crystals of calcium oxalate. Stem rounded with slightly protruding ribs. The fascicules of the central axial cylinder are open collateral, arranged in a circle, with a sclerenchyma lining on the side of the phloem, naked, with a two-layered lamellar collenchyma.

As emphasized, attention should be paid for exploitation to the presence of different biological groups that may provide synergistic health effects of plant extracts. The presence of diversified compounds of phytochemical composition, and their contemporary presence of structurally different biologically active groups in different extracts (usually used in casual) were likely to determine different responses to the antioxidant assays.

The presence of compounds belonging to different biologically active groups can provide a synergistic effect of plant extracts on the state of health. In our case, the phytochemical composition of meadow clover grass has become a guarantee of antioxidant activity of extracts from this plant material. And the best results we found in the aqueous extract with a ratio of plant material: extractant 1: 5.

In conclusion, the impact of our study lies in the fact that the optimal network can be obtained when the available prior knowledge only provides information on the overall relationship across data (Fig. 6). Consequently, a network fitted to partial priors can be used to enrich the knowledge with new, previously unknown interactions, or to rectify incorrect links, and can therefore serve as a valuable tool to infer biological interactions. Application of boxplots to investigate the pharmacognostic properties of Trifolii repens extracts can show similarities, differences, trends, and irregularities among groups, which help better understand their characteristics.

Conflict of interest

The authors hereby declare that the work in the paper has no competing interests of any type to anybody or any institution.

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