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Comparative Phytochemical and Densitometric Analyses of *Capsella bursa-pastoris* and *Trifolium pratense*: Implications for Natural Anticancer Therapies

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Abstract

The growing interest in natural-based products as complementary and alternative therapies reflects the need for safer and more acceptable anticancer agents compared to conventional chemotherapy. This study aimed to analyze the phytochemical composition and anticancer potential of *Capsella bursa-pastoris* and *Trifolium pratense*, two species that are traditionally used in herbal medicine. Phytochemical screening was performed using thin-layer chromatography (TLC), which allowed for the qualitative identification of the main bioactive compounds. Densitometric analysis confirmed the presence of nine main compounds that absorb UV radiation, mainly polyphenols and isoflavones. The results showed that *Trifolium pratense* has a richer and more complex phytochemical profile compared to *Capsella bursa-pastoris*, with higher concentrations of phenolic compounds and isoflavones. These constituents are recognized for their antioxidant and chemoprotective properties, which support cellular defense against oxidative stress and contribute to potential anticarcinogenic activity, particularly in skin-related processes. The TLC method has proved to be an effective preliminary tool for differentiating phytochemical profiles and standardizing plant extracts. In conclusion, *Trifolium pratense* is a promising candidate for the development of standardized phytotherapeutic formulations. Future studies should focus on the isolation and characterization of its active compounds, the evaluation of biological mechanisms, and the formulation of topical products with validated efficacy and safety.

Keywords: Trifolium pratense*;* Capsella bursa-pastoris*; phytochemical analysis; anticancer activity; thin-layer chromatography (TLC)*

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1. Introduction

The growing interest in using complementary and alternative medicines reflects the need for safer and better-tolerated treatment options, especially considering the drawbacks of conventional chemotherapy. The phytochemical compounds found in plants, such as those in leaves, stems, roots, bulbs, and bark, serve as crucial starting points for discovering and developing new anticancer drugs. Bioactive compounds can be used directly in traditional therapies as tinctures, teas, or extracts and provide a clear foundation for synthesizing molecules with improved efficacy and safety. However, the variability in the preparation methods and chemical composition of natural products underscores the need for thorough phytochemical analysis, which enables accurate identification of active molecules, standardization of dosage, and design of effective pharmacological forms [1].

To understand how these plants exert their anticancer effects and to facilitate subsequent in vitro testing, a rigorous phytochemical analysis of the active compounds in *Capsella bursa-pastoris* and *Trifolium pratense* is essential.

Capsella bursa-pastoris, a perennial plant from the *Brassicaceae* family, has been used medicinally for over 8000 years, with archeological evidence dating back to the Neolithic period (Catalhoyuk, Turkey). Traditionally, it has been used as a hemostatic, vasoconstrictor, and antipyretic, as well as for treating edema, hypertension, and kidney or nervous system disorders. Modern ethnopharmacological studies have confirmed that is has multiple biological effects, including anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, sedative, and anticancer properties [2, 3]. Its pharmacological potential is closely linked to its complex phytochemical profile, which includes flavonoids, phenolic acids, biogenic amines, alkaloids, vitamins, minerals, and essential oils. Due to its unique chemical composition, *C. bursa-pastoris* is a promising candidate for cancer research and the development of standardized herbal products [4]. *Trifolium pratense* (red clover) belongs to the *Fabaceae* (Leguminosae) family, one of the most important and studied plant families due to its economic and agricultural value, as it includes many species used for food, feed, and medicine [5]. The pharmacological potential of *Trifolium pratense* is strongly connected to its rich phytochemical profile, which includes isoflavones, flavonoids, phenolic acids, vitamins, and minerals. These bioactive compounds give the plant antioxidant, anti-inflammatory, estrogenic, and chemoprotective effects, supporting DNA protection against oxidative stress and stimulating endogenous antioxidant enzymes. Because of its diverse and potent chemical composition, *T. pratense* is a promising candidate for anticancer research and the development of standardized herbal products [6]. Although natural products are often viewed as safe, they can pose toxic risks if not properly identified or if doses are not controlled. Variability in chemical composition between batches and the lack of official regulation make it difficult to assess the efficacy and safety of herbal therapies [7]. In this context, phytochemical research becomes essential, not only for identifying and standardizing active components but also for guiding in vitro studies that investigate anticancer mechanisms [8]. Additionally, the controlled use of natural resources through cultivation or semi-synthetic methods helps conserve valuable medicinal species and supports the sustainability of long-term research. Therefore, phytochemical analysis is a fundamental step in developing effective, safe, and standardized herbal therapies and in investigating anticancer effects across experimental and clinical models [9].

This study aims to contribute to the understanding of the phytochemical composition and anticancer potential of these two species, supporting their future application in natural-product-based cancer therapy.

2. Materials and Methods

The analytical methods used in this study included: thermogravimetric determination of water content, evaluation of total antioxidant capacity using the CUPRAC method, determination of total polyphenol content in *Trifolium pratense* and *Capsella bursa-pastoris* extracts, as well as thin-layer chromatography (TLC) analysis to qualitatively highlight the main classes of bioactive compounds with relevant potential in skin cancer.

2.1. Obtaining Extracts

A static maceration method was used to obtain the plant extracts. Ten grams of dried and crushed plant material was subjected to extraction with 50 mL of 96% ethyl alcohol at room temperature in the dark for a period of 7 days to prevent the degradation of light-sensitive compounds.

2.2. Thermogravimetric Analysis of the Water Content in the Analyzed Plant Material Samples

The thermogravimetric analysis of plant samples was performed using a Sartorius thermobalance, a device that allows real-time monitoring of the dehydration process by continuously weighing the sample during heating. This method provides accurate information on the mass loss associated with water removal, which is essential for the preliminary characterization of the plant material used in phytochemical analyses. The measurement accuracy of the equipment is 0.1% for samples with a mass greater than 1 g and 0.02% for samples with a mass greater than 5 g [10]. The determination was performed by weighing 1 g of crushed plant material from each sample. The thermobalance was set to a maximum temperature of 110 °C, which was sufficient for complete water evaporation without significant thermal degradation of sensitive organic compounds. The dehydration process was controlled, and continuous weighing allowed automatic recording of mass variation over time. The evolution of weight loss, correlated with the gradual increase in temperature, provided information on the thermal stability and moisture content of the plant material.

2.3. Thin-Layer Chromatography (TLC) Analysis

A starting line was drawn on each chromatographic plate with a pencil and a ruler 1 cm from the base of the plate. The samples were applied to this line using a 1 μL microsyringe or a fine capillary tube in the form of small circular spots to avoid overlap during migration. The samples analyzed were the alcoholic extract of *Trifolium pratense* (red clover) and

the alcoholic extract of *Capsella bursa-pastoris* (shepherd's purse). After applying the samples, the plates were placed in a vertical developing chamber, previously saturated with solvent vapors, to ensure uniform migration of the mobile phase. To optimize separation, several elution systems were tested, as shown in **Table 1**, containing solvents of different polarities, to obtain the clearest possible chromatographic profile of the compounds present.

Table 1. Elution systems tested for *Trifolium pratense* and *Capsella bursa-pastoris* extracts.

No.	Elution System	Volume Ratio
1	Chloroform:Methanol:Water	8:1:1
2	Ethyl acetate:Methanol	4:1
3	Ethyl acetate:Hexane	9:1
4	Chloroform:Methanol	9.5:0.5
5	Acetone:Hexane	7:3
6	Hexane:Ethyl ether:Acetic acid	9.5:0.5

After completion of the migration process (when the solvent front reached approximately 1 cm from the upper edge of the plate), the plates were removed from the developing chamber using tweezers and dried for several minutes in an oven at 100 °C. After complete drying, the chromatograms were analyzed by UV irradiation using a Fisher Bioblock Scientific spectrophotometer at wavelengths of 256 nm for viewing aromatic, phenolic, and flavonoid compounds, and 365 nm for identifying flavonoids and other naturally fluorescent compounds. The results obtained were photographed and interpreted qualitatively by comparing the intensity and position of the spots between the different elution systems. These data provide an overview of the phytochemical composition of the tested extracts, which is useful in the preliminary stage of evaluating their anticancer potential.

2.4. Determination of Total Antioxidant Capacity by the CUPRAC Method

The total antioxidant capacity was determined using the CUPRAC (Cupric Reducing Antioxidant Capacity) method, which is based on the ability of antioxidant compounds in plant extracts to reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}) in the presence of the ligand neocuproine, forming a colored complex measurable spectrophotometrically at 450 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an antioxidant soluble in both water and fats, was used as a reference standard. To determine antioxidant activity, 1 mL of 0.01 M copper chloride solution, 1 mL of alcoholic neocuproine solution (7.5×10^{-3} M), and 1 mL of acetate buffer solution (pH 7.0) were mixed. To this mixture, 1.1 mL of the sample, standard (Trolox), or blank solution was added. The solutions were homogenized and kept at room temperature for 30 min, after which the absorbance was determined at 450 nm using a UV-Vis spectrophotometer. The total antioxidant capacity was expressed in μmol Trolox equivalents per gram of sample (μmol TE/g).

2.5. Determination of Total Polyphenol Content by Photometric Method (Folin–Ciocalteu)

The total polyphenolic compound content was determined using the Folin–Ciocalteu colorimetric method based on the reducing properties of polyphenols. These polyphenols reduced the Folin–Ciocalteu reagent, forming a blue-colored complex with maximum absorption at 750 nm. The intensity of the color was proportional to the total concentration of polyphenols in the sample. The alcoholic extracts obtained from the plant material were diluted accordingly. From each sample, 0.5 mL of extract was taken, to which 2.5 mL of Folin–Ciocalteu reagent (1:10, v/v) and 2 mL of 7.5% sodium carbonate solution (m/v) were added. The mixture was homogenized and incubated at room temperature for 30 min, after which the absorption was measured spectrophotometrically at 750 nm. Gallic acid was used as a standard in concentrations of 0–0.7 μM/mL to construct the calibration curve. The results were expressed as mg gallic acid equivalents (GAE) per 100 g sample.

2.6. Densitometric Analysis of TLC Chromatograms

Densitometric analysis was performed using the CAMAG TLC Scanner 3 densitometer, controlled by WinCATS software. Densitometry enables the quantification of substances separated on a chromatographic plate by measuring the intensity of the optical signal (absorption or fluorescence) at each position on the plate. This method provides an accurate and reproducible assessment of the relative content of bioactive compounds, complementing the visual observations obtained by thin-layer chromatography. To identify and characterize the separation zones, the Rf (retention factor) values were

determined for each detected compound. The Rf value expresses the ratio between the distance traveled by a substance on the plate (*Xs*, the distance between the start line and the center of the spot) and the distance traveled by the solvent front (*XD*):

$$Rf = \frac{Xs}{XD}$$

The *Rf* values obtained provide specific information about the migration of the compounds and allow for comparison between the analyzed extracts (*Trifolium pratense* and *Capsella bursa-pastoris*) to identify similarities or differences in phytochemical composition.

3. Results and Discussions

3.1. Thermogravimetric Analysis

From the analysis of the data presented in **Table 2**, it was found that the dehydration process took place in a shorter time for *Capsella bursa-pastoris* (approximately 9 min) compared to *Trifolium pratense*, for which complete drying took approximately 14 min. Both species have a high water content of over 90%, which indicates an advanced degree of hydration of plant tissues (**Figure 1**), as evidenced by the thermogravimetric curves (**Figure 2**). The differences in dehydration time can be explained by the morphological and structural characteristics of the plant material, such as tissue thickness and density, hygroscopic substance content, or the proportion of different cell fractions.

Table 2. Results of thermogravimetric analysis to determine the water content in the plant samples analyzed.

Trifolium pratense		***Capsella bursa-pastoris***	
Time (min)	**U%**	**Time (min)**	**U%**
0	0.10	0	0.10
1	10.75	1	11.65
2	41.28	2	45.27
3	57.18	3	59.18
4	66.67	4	68.37
5	72.15	5	74.25
6	77.13	6	78.13
7	81.21	7	84.21
8	84.59	8	88.19
9	87.08	9	90.08
10	88.85		
11	90.43		
12	91.81		
13	92.69		
14	92.97		

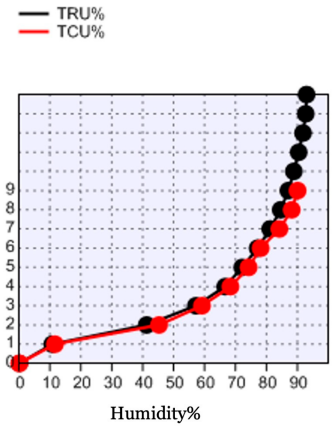


Figure 1. Thermogravimetric analysis—graphical representation of mass loss (water content) as a function of analysis time.

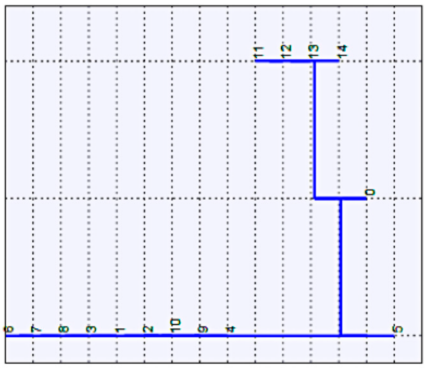


Figure 2. Thermogravimetric analysis—graphical cluster representation of mass loss variation as a function of analysis time.

3.2. Total Antioxidant Capacity (TAC) and Total Polyphenol Content (PF) in Plant Sample Extracts (μmol Trolox/g Sample)

The total antioxidant capacity (TAC) and total polyphenol content (TP) were determined for extracts of *Trifolium pratense* and *Capsella bursa-pastoris* using hydroalcoholic solutions of different concentrations and varying extraction times. The CUPRAC method was applied to determine total antioxidant activity, and total polyphenol content was determined using the Folin–Ciocalteu method. The obtained results are presented in **Table 3**.

Table 3. Total antioxidant capacity (TAC) and total polyphenol content (TP) of *Trifolium pratense* and *Capsella bursa-pastoris* extracts, determined by the CUPRAC and Folin–Ciocalteu methods, respectively.

Plants	Alcoholic Solution	Extraction Time (hour)	TAC (μmol Trolox/g sample)	TPC (mg gallic acid/100 g DW)
Trifolium pratense	20% ethanol solution	24	3.110	10.710
Capsella bursa-pastoris	20% ethanol solution	24	3.010	8.470
Trifolium pratense	20% ethanol solution	144	1.370	9.660
Capsella bursa-pastoris	20% ethanol solution	144	1.080	7.320
Trifolium pratense	96° ethanol	1440	1.305	1.910
Capsella bursa-pastoris	96° ethanol	1440	0.840	0.800

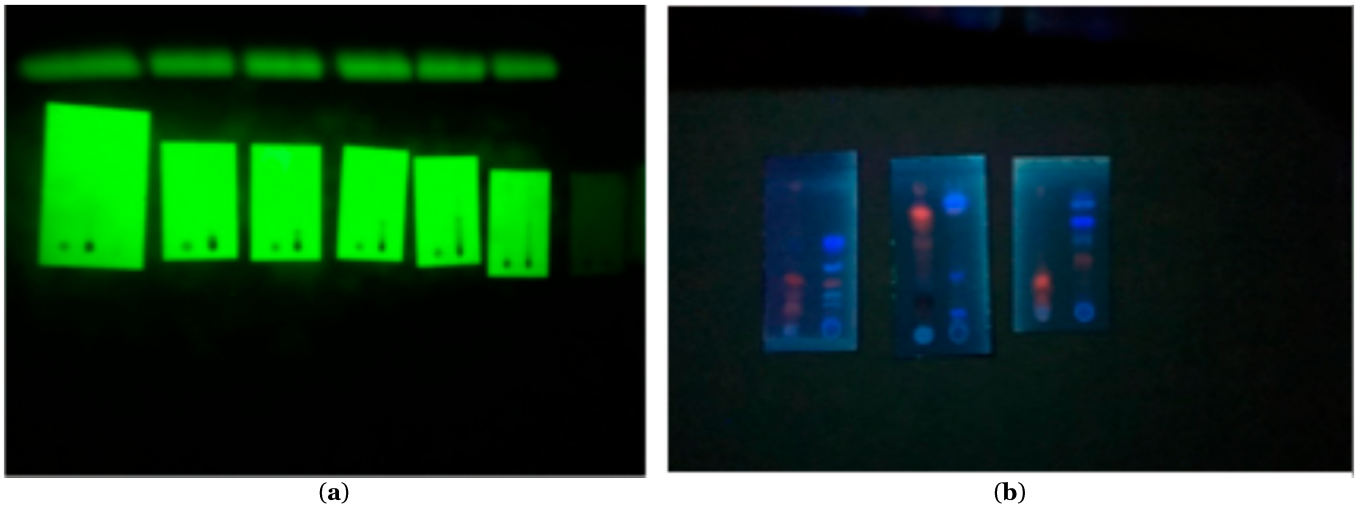
TAC = total antioxidant capacity, expressed as μmol Trolox equivalents per gram of sample; TPC = total polyphenol content, expressed as mg gallic acid equivalents per 100 g dry weight (DW).

The analysis of the values obtained highlights notable differences between the two plant species regarding both solvent type and extraction duration. It shows that, at an alcohol concentration of 20% and an extraction time of 24 h, *Trifolium pratense* exhibits the highest values for total antioxidant capacity (3110 μmol Trolox/g DM) and polyphenol content (10,710 mg/L gallic acid/100 g DM), surpassing the corresponding values for *Capsella bursa*-pastoris (3010 μmol

Trolox/g DM and 8470 mg/L gallic acid/100 g DM). In the case of extended extractions (144–1440 h) and solvents with high alcohol concentrations (96°), a gradual decrease in antioxidant activity and total polyphenol content is observed, a phenomenon attributed to thermal degradation and oxidation of phenolic compounds sensitive to prolonged extraction conditions [11]. The differences between the two plants can be explained by their unique phytochemical profiles. *Trifolium pratense* contains isoflavones (genistein, daidzein, formononetin, biochanin A), anthocyanins, and phenolic acids, all known for their potent antioxidant activity [12]. Conversely, *Capsella bursa*-pastoris contains peptides, glycosides, and unsaturated fatty acids, which serve different biological functions and contribute less to free radical neutralization [13]. The findings confirm that moderate solvent polarity (20% ethanol) and a short extraction duration (24 h) are optimal for producing an extract rich in phenolic compounds with high antioxidant activity.

3.3. Thin-Layer Chromatography (TLC)

Following the described TLC method, distinct chromatographic profiles were obtained for the alcoholic extracts of *Trifolium pratense* and *Capsella bursa-pastoris*(**Figure 3**). The qualitative analysis aimed to achieve the preliminary identification of phenolic, flavonoid, and isoflavonoid compounds, based on their chromatographic behavior and fluorescence under UV light. Among the tested elution systems (**Table 1**), the best results were obtained with the following combinations: for *Capsella bursa-pastoris*, ethyl acetate:methanol:water (8:1:1, *v*/*v*/*v*), and for *Trifolium pratense*, ethyl acetate:hexane (9:1, *v*/*v*). In the case of *Capsella bursa-pastoris*, UV analysis at 256 nm revealed three visible spots, and after spraying the plate with ammonium molybdate and sulfuric acid, four to five distinct spots appeared, confirming the presence of phenolic and glycosidic compounds. The color reaction (blue–brown) indicated that most compounds were reduced oxidants with moderate antioxidant potential. For the *Trifolium pratense* extract, the ethyl acetate:hexane system allowed clear separation of apolar and semipolar compounds. Under UV radiation at 256 nm, three spots were observed, and at 365 nm, there were five fluorescent areas, corresponding to isoflavones and other polyphenolic compounds. The fluorescence intensity and contrast corroborate a higher concentration of conjugated aromatic compounds, supporting the high antioxidant activity previously determined in Section 3.2.



(**a**) (**b**)

Figure 3. Comparative TLC profiles of *Trifolium pratense* and *Capsella bursa-pastoris* extracts observed under UV light: (**a**) at 256 nm and (**b**) at 356 nm.

Comparative analysis of chromatographic profiles reveals greater phytochemical complexity in *Trifolium pratense* compared to *Capsella bursa-pastoris*. The higher number of spots and increased fluorescence intensity suggest a high content of isoflavones, flavonoids, and phenolic derivatives, known for their role in reducing oxidative stress and inhibiting skin tumor processes [12]. *Capsella bursa-pastoris* extracts showed a simpler profile with a predominance of polar compounds (glycosides, peptides), indicating a moderate but complementary antioxidant potential [4].

3.4. Densitometry

3.4.1. *Trifolium pratense*

After obtaining the chromatographic profiles by TLC, the plates were subjected to densitometric analysis using the CAMAG TLC Scanner to accurately assess the distribution and intensity of the separated compounds. The densitometric

analysis was performed at wavelengths between 200 and 400 nm to capture the full absorption spectrum of phenolic, flavonoid, and isoflavonoid compounds. **Figure 4** illustrates the three-dimensional (3D) image of the densitogram obtained for the ethanolic extract of *Trifolium pratense*, developed in the ethyl acetate:hexane = 9:1 (*v*/*v*) elution system. The densitogram shows the variation in absorption intensity (A.U.) as a function of the retention factor (Rf) value corresponding to each separately chromatographed compound.

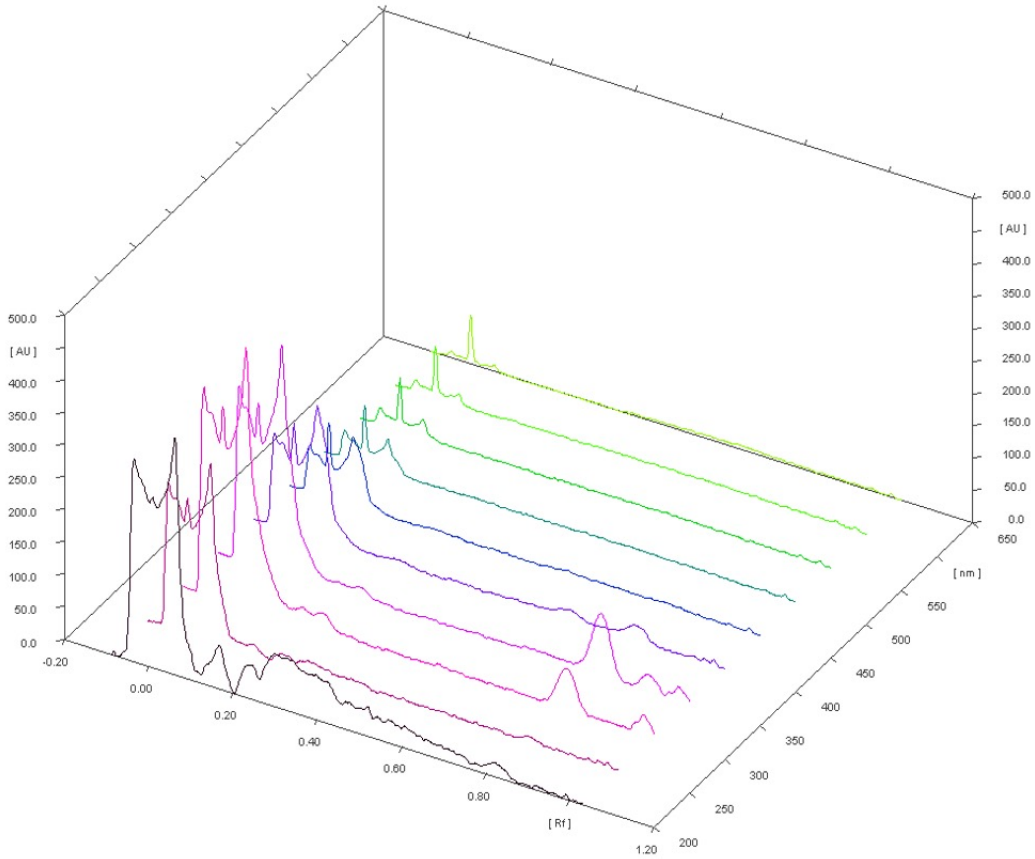


Figure 4. Three-dimensional (3D) representation of the densitometric analysis for the alcoholic extract of *Trifolium pratense,* developed in the ethyl acetate:hexane = 9:1 (*v*/*v*) elution system.

Densitometric analysis revealed nine distinct peaks corresponding to the main compounds present in red clover extract. The Rf values, recorded between 0.07 and 0.86, indicated a uniform distribution of compounds of varying polarity, suggesting a complex phytochemical composition. The data obtained are presented in **Table 4**.

Densitometric analysis (**Figures 5–9**) confirms the effective separation of bioactive compounds from *Trifolium pratense* extract, with each peak representing a compound with specific absorption in the UV–Vis range (**Tables 5–8**). The most intense signals (peaks 1 and 2) have maximum absorption values of 257.9 and 301.2 AU, representing approximately 64% of the total chromatographic area, which suggests the predominant presence of the isoflavones genistein and daidzein, phenolic compounds with strong antioxidant activity. The intermediate Rf values (0.26–0.46) probably correspond to flavonoids and anthocyanins, which contribute significantly to the total antioxidant capacity. The final peak (Rf ≈ 0.78–0.82) can be attributed to nonpolar compounds (sterols and triterpenes), present in a small proportion (≈2%). The distribution of the nine peaks confirms the polycomponent nature of red clover extract and supports the results in previous Sections 3.2 and 3.3, indicating high phytochemical diversity and complex antioxidant potential specific to plants with multiple biological activities.

Table 4. Densitometric parameters of *Trifolium pratense* extract.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P (RF)	End H (AU)	Area (AU)	Area %
1	−0.07	0.4	−0.03	257.9	28.67	0.00	108.3	6181.5	28.85
2	0.02	205.3	0.06	301.2	33.48	0.11	12.9	8119.1	35.27
3	0.12	10.3	0.17	55.1	6.12	0.20	0.9	1234.3	5.36
4	0.20	1.1	0.25	42.9	4.77	0.26	27.5	976.1	4.24
5	0.26	27.6	0.30	66.3	7.37	0.32	63.6	1717.7	7.46
6	0.32	63.9	0.33	65.6	7.29	0.36	55.9	1435.8	6.24
7	0.38	54.1	0.40	56.4	6.26	0.46	29.3	2198.9	9.55
8	0.49	30.7	0.52	37.1	4.13	0.53	24.0	675.2	2.93
9	0.78	7.1	0.82	17.2	1.91	0.86	3.3	479.6	2.08

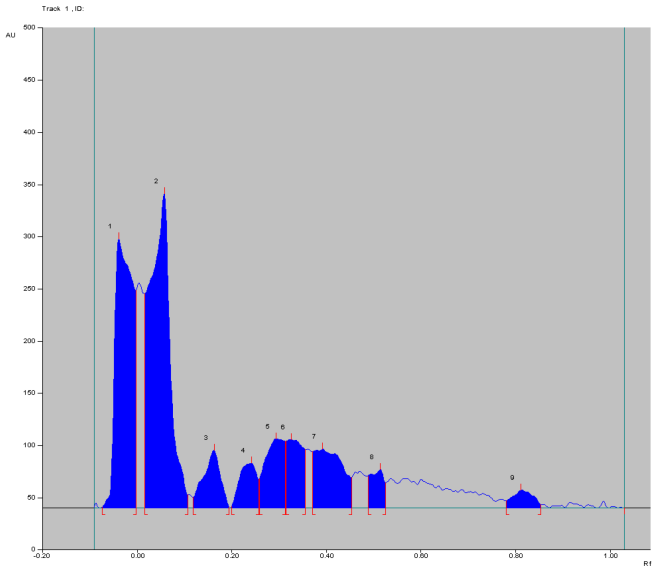


Figure 5. Densitogram showing the nine peaks detected by the densitometer on the chromatographic plate, obtained after separating the *Trifolium pratense* extract in the ethyl acetate:hexane = 9:1 (*v*/*v*) elution system, at different wavelengths in the range 200–650 nm. The blue area represents the densitometric profile of the TLC plate (UV–Vis absorbance); red vertical lines represent integrated peaks corresponding to separated compounds.

Table 5. The retention factor (Rf) values and peak areas are highlighted for the *Trifolium pratense* extract developed in an ethyl acetate:hexane (9:1, *v*/*v*) elution system at a wavelength of 200 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P (RF)	End H (AU)	Area (AU)	Area %
1	−0.07	0.5	−0.04	207.1	30.36	0.00	58.9	4734.7	34.11
2	0.00	163.1	0.01	193.6	28.38	0.02	52.7	1871.4	13.48
3	0.02	153.4	0.06	253.8	37.21	0.13	10.5	6715.2	48.38
4	0.15	10.4	0.17	13.9	2.04	0.20	1.4	237.1	1.71
5	0.21	0.4	0.24	13.8	2.02	0.26	7.0	320.5	2.31

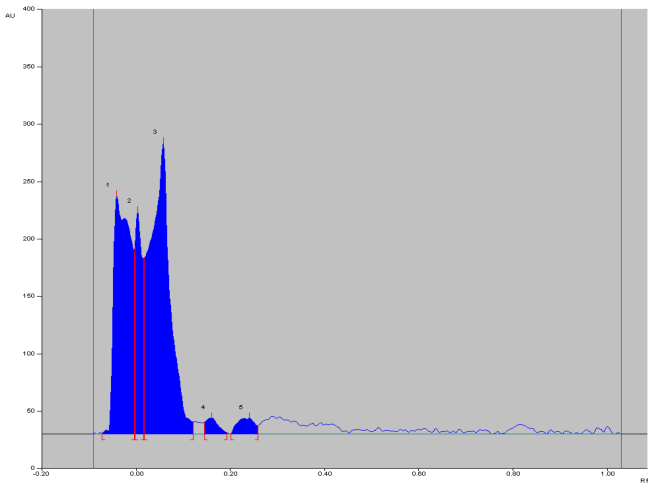


Figure 6. Densitogram recorded at 200 nm, corresponding to the chromatographic plate resulting from the separation of the *Trifolium pratense* extract in the ethyl acetate:hexane = 9:1 (*v*/*v*) elution system. The blue area represents the densitometric profile of the TLC plate (UV–Vis absorbance); red vertical lines represent integrated peaks corresponding to separated compounds.

Table 6. The retention factor (Rf) values and peak areas are highlighted for the *Trifolium pratense* extract developed using an ethyl acetate:hexane (9:1, *v*/*v*) elution system at a wavelength of 250 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P (RF)	End H (AU)	Area (AU)	Area %
1	-0.06	0.5	-0.04	271.0	28.79	0.00	102.9	6179.7	26.77
2	0.00	210.4	0.01	253.5	26.94	0.02	98.5	228.9	10.38
3	0.02	198.6	0.06	340.3	36.15	0.17	16.2	1098.4	51.15
4	0.22	17.2	0.24	23.7	2.51	0.28	5.3	577.8	2.69
5	0.78	4.2	0.83	52.8	5.61	0.88	0.2	1507.9	7.02

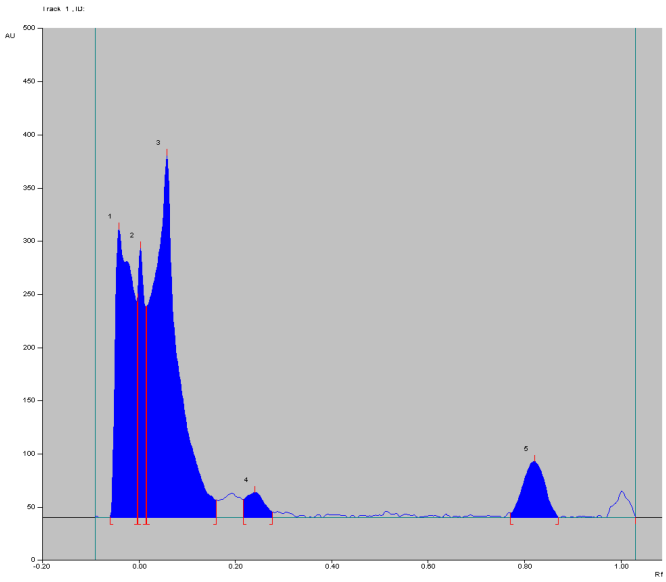


Figure 7. Densitogram recorded at 300 nm, corresponding to the chromatographic plate resulting from the separation of the *Trifolium pratense* in the ethyl acetate:hexane = 9:1 (*v*/*v*) elution system, highlighting the five peaks detected by the densitometer, which correspond to the chromatographically separated compounds.

Table 7. The retention factor (Rf) values and peak areas are highlighted for the *Trifolium pratense* extract, which was developed using an ethyl acetate:hexane (9:1, *v*/*v*) elution system at a wavelength of 350 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P (RF)	End H(AU)	Area (AU)	Area %
1	−0.06	0.9	−0.04	263.2	6.19	0.00	92.6	5972.0	25.59
2	0.00	199.0	0.01	244.8	24.36	0.02	95.5	1933.3	8.28
3	0.02	185.9	0.06	343.5	34.18	0.17	10.6	11511.8	49.33
4	0.22	11.7	0.25	17.4	1.73	0.26	4.6	402.1	1.72
5	0.75	3.7	0.82	93.2	9.27	0.88	0.2	2704.0	11.50
6	0.90	0.40	0.93	23.1	2.30	0.97	0.4	532.9	2.26
7	0.96	0.4	1.01	19.9	1.96	1.02	12.0	280.5	1.20

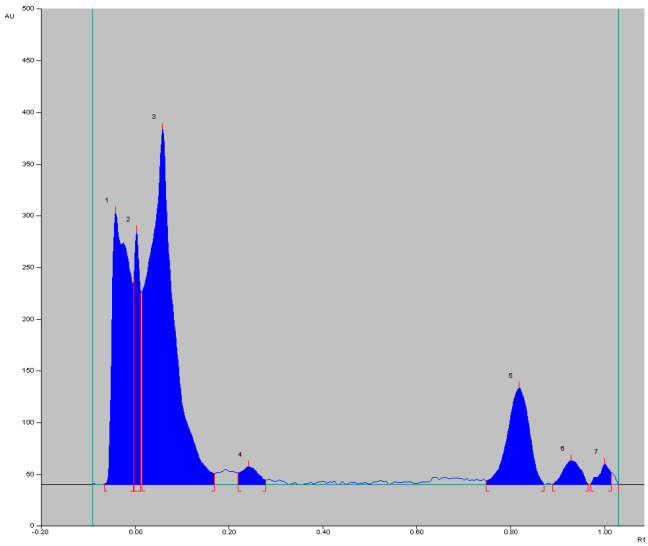


Figure 8. Densitogram recorded at 350 nm, corresponding to the chromatographic plate obtained after separation of the *Trifolium pratense* (TR) extract in the ethyl acetate:hexane (9:1, *v*/*v*) elution system. Seven peaks detected by the densitometer are highlighted, corresponding to the chromatographically separated compounds.

Table 8. Retention factor (Rf) values and peak areas are highlighted for *Trifolium pratense* extract using the ethyl acetate:hexane (9:1, *v*/*v*) elution system at a wavelength of 400 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P (RF)	End H (AU)	Area (AU)	Area %
1	−0.07	0.2	-0.04	150.3	25.51	−0.00	17.3	3266.7	24.01
2	−0.00	115.0	0.01	176.9	30.02	0.02	11.6	1644.4	12.09
3	0.02	111.9	0.06	218.4	37.07	0.15	9.5	7367.9	54.6
4	0.63	8.1	0.67	17.3	2.93	0.71	4.7	559.7	4.11
5	0.78	8.9	0.82	26.3	4.47	0.86	6.4	766.4	5.83

Analysis of the densitograms and retention factor (Rf) values for the *Trifolium pratense* extract revealed significant variations depending on the detection wavelength. The most complex separations were observed at 300 nm and 350 nm, where five and seven distinct peaks were identified, respectively, indicating a higher presence of detectable active compounds in the near-visible range of the UV spectrum. The higher Rf values (0.82 and 0.93) recorded at these wavelengths suggest greater chromatographic mobility of the compounds, possibly associated with flavonoid or isoflavonoid substances characteristic of red clover extract. At longer wavelengths (400 nm), the number of peaks detected decreased, indicating a decrease in densitometric response and a reduced presence of compounds that absorb in this range.

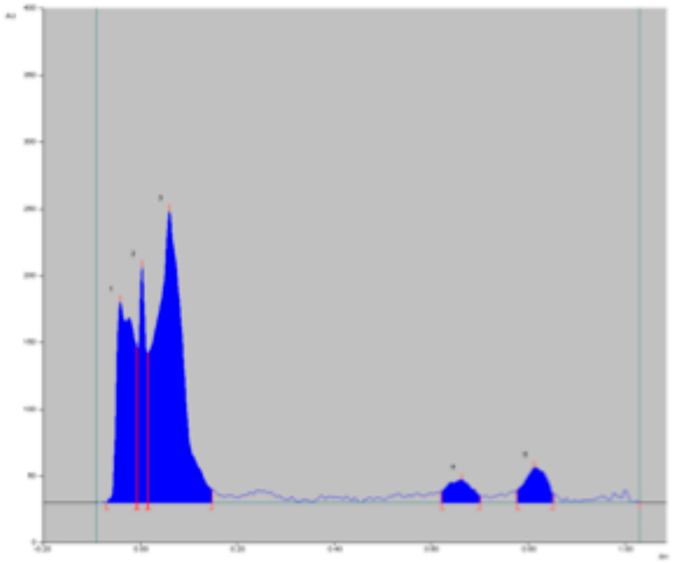


Figure 9. Densitogram recorded at 400 nm, corresponding to the chromatographic plate obtained after separation of the *Trifolium pratense* extract in the ethyl acetate:hexane = 9:1 (*v*/*v*) elution system. Five peaks detected by the densitometer are highlighted, corresponding to the chromatographically separated compounds.

3.4.2. *Capsella bursa-pastoris*

Densitometric analysis of *Capsella bursa-pastoris* extract developed in the AcOEt:MeOH:H_2O (8:1:1, *v*/*v*/*v*) system revealed a complex profile of chromatographic compounds. At a wavelength of 200 nm, the 3D densitogram (**Figure 10**), together with the Rf values indicated in **Tables 9–12**, shows the presence of nine distinct peaks, suggesting a wide variety of secondary metabolites with different polarities. The corresponding chromatographic profiles are presented in **Figures 11–13**. Peaks with low Rf values (−0.05–0.03) correspond to more polar compounds with high absorption intensities (414–521 A.U.) and a significant contribution to the total area (14–47%), indicating that these compounds are predominant in the extract. On the other hand, compounds with higher Rf values (>0.5) have relatively low intensity and area (<1%), suggesting a lower concentration of more nonpolar substances. These results confirm that densitometric analysis at 200 nm allows for the effective detection of most phenolic and flavonoid compounds present in the extract. The 3D image provides a clear representation of the distribution of compounds according to Rf and absorption, facilitating comparison between major and minor components and providing support for their qualitative and quantitative identification.

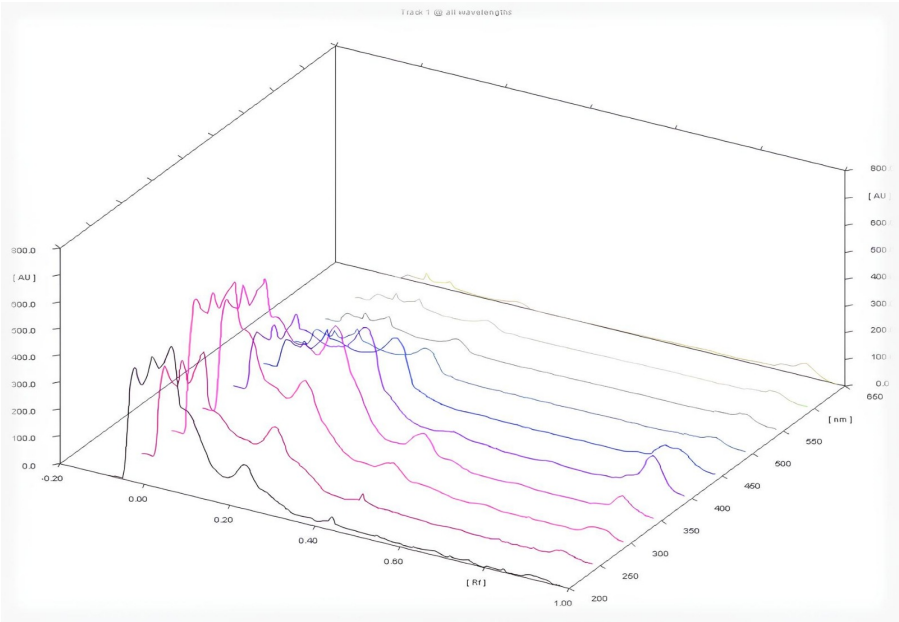


Figure 10. 3D representation of the extract from *Capsella bursa-pastoris* obtained by densitometric analysis and developed in the AcOEt:MeOH:H_2O = 8:1:1 elution system. The spectrum was recorded at wavelengths between 200 and 650 nm, and the 3D diagram illustrates the Rf values of the separated substances.

Table 9. Retention factor (Rf) values and peak areas for *Capsella bursa-pastoris* extract developed in the ethyl acetate:methanol: water = 8:1:1 ($v/v/v$) elution system at a wavelength of 200 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P (RF)	End H (AU)	Area (AU)	Area %
1	−0.05	1.8	−0.02	414.5	24.82	−0.01	57.4	7146.4	14.59
2	−0.01	359.9	0.02	467.9	28.01	0.03	32.5	8876.5	18.13
3	0.03	433.9	0.06	521.2	31.20	0.19	35.5	23,015.0	47.00
4	0.19	97.0	0.23	159.2	9.53	0.35	22.7	7557.5	15.43
5	0.41	17.6	0.44	47.1	2.82	0.49	16.8	1092.9	2.23
6	0.53	14.0	0.55	20.0	1.20	0.58	8.9	434.9	0.89
7	0.66	9.8	0.68	14.6	0.88	0.71	2.3	289.5	0.59
8	0.74	5.8	0.76	11.8	0.71	0.79	0.2	172.2	0.35
9	0.83	1.5	0.92	14.1	0.84	0.96	0.8	386.8	0.79

Table 10. The retention factor (Rf) values and peak areas highlighted for the *Capsella bursa-pastoris* extract developed in the ethyl acetate:methanol:water = 8:1:1 ($v/v/v$) elution system at a wavelength of 250 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P (RF)	End H (AU)	Area (AU)	Area %
1	−0.05	0.3	−0.02	334.1	23.77	−0.00	79.2	6224.8	15.26
2	0.00	274.3	0.02	369.9	26.32	0.03	34.4	5164.6	12.66
3	0.03	295.6	0.07	418.3	29.77	0.16	41.2	16,756.8	41.08
4	0.18	133.6	0.23	213.3	15.18	0.37	17.6	11,164.2	27.37
5	0.40	14.5	0.44	48.0	3.42	0.48	11.3	955.0	2.34
6	0.88	3.1	0.91	21.6	1.54	0.96	2.1	522.7	1.28

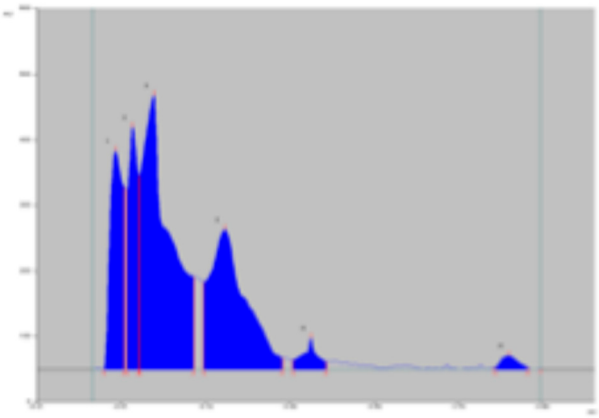


Figure 11. Densitogram highlighting the six peaks detected by the densitometer on the chromatographic plate resulting from the separation of the *Capsella bursa-pastoris* extract using the elution system AcOEt: MeOH:H2O = 8:1:1 at a wavelength of 250 nm.

Table 11. The retention factor (Rf) values and peak areas highlighted for the *Capsella bursa-pastoris* extract developed in the ethyl acetate:methanol:water = 8:1:1 (*v*/*v*/*v*) elution system at a wavelength of 350 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P(RF)	End H (AU)	Area (AU)	Area %
1	-0.05	0.3	-0.02	405.4	15.16	-0.01	84.9	7151.1	10.03
2	0.00	383.0	0.02	469.5	17.56	0.03	105.7	6858.4	9.62
3	0.03	407.3	0.07	511.8	19.14	0.09	31.3	12,998.0	18.23
4	0.09	381.8	0.09	384.3	14.37	0.14	107.7	10,800.3	15.14
5	0.15	307.8	0.16	311.8	11.66	0.19	86.6	6978.8	9.79
6	0.19	287.2	0.23	403.8	15.10	0.36	45.9	20,454.2	28.68
7	0.38	44.7	0.44	101.5	3.80	0.49	31.0	3803.7	5.33
8	0.49	30.7	0.50	32.6	1.22	0.56	4.9	804.3	1.13
9	0.85	8.0	0.91	52.8	1.97	0.97	4.5	1467.8	2.06

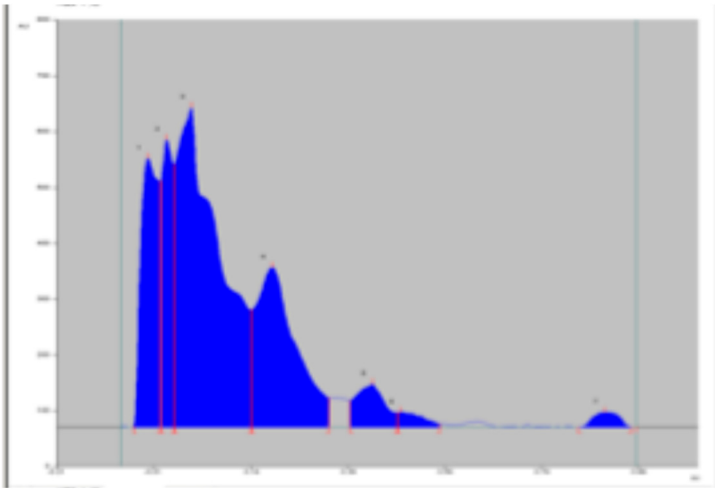


Figure 12. Densitogram recorded at 350 nm corresponding to the chromatographic plate obtained after separation of the *Capsella bursa-pastoris* extract in the ethyl acetate:methanol:water = 8:1:1 (*v*/*v*/v) elution system. Seven peaks detected by the densitometer are highlighted, corresponding to the chromatographically separated compounds.

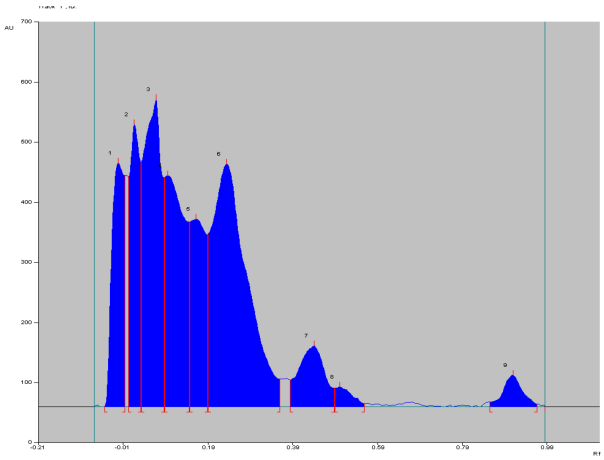


Figure 13. Densitogram recorded at 400 nm, corresponding to the chromatographic plate obtained after separation of *the Capsella bursa-pastoris* extract in the ethyl acetate:methanol:water = 8:1:1 (*v*/*v*/*v*) elution system. Nine peaks detected by the densitometer are highlighted, corresponding to the chromatographically separated compounds.

Table 12. Retention factor (Rf) values and peak areas for *Capsella bursa-pastoris* extract developed in the ethyl acetate:methanol: water = 8:1:1 ($v/v/v$) elution system at a wavelength of 400 nm.

Peak	Start P (Rf)	Start H (AU)	Max P (Rf)	Max H (AU)	Max %	End P RF)	End H (AU)	Area (AU)	Area %
1	-0.06	0.1	-0.02	112.4	14.55	0.01	95.0	2586.5	9.40
2	0.01	97.3	0.02	121.3	15.70	0.03	100.2	1291.8	4.69
3	0.03	1005.5	0.07	317.4	23.84	0.14	81.8	8404.6	30.53
4	0.14	132.1	0.23	329.2	28.05	0.38	3.7	12,050	43.78
5	0.77	2.5	0.80	30.4	1.84	0.81	12.0	241.8	0.88
6	0.81	12.3	0.87	120.1	8.07	0.88	59.1	1448.0	5.26
7	0.88	59.2	0.89	215.4	7.95	0.96	1.0	1503.4	5.46

Densitometric analysis of the *Capsella bursa-pastoris* extract, developed using an ethyl acetate:methanol:water (8:1:1, $v/v/v$) elution system, revealed a complex chromatographic profile characteristic of plant extracts rich in phenolic and flavonoid compounds. At 200 nm, many polar compounds with high absorption intensities were observed, typical of phenolic acids and glycosylated flavonoids, indicating their dominance in the extract. At 250 nm, moderate absorptions confirmed the presence of conjugated aromatic compounds, especially substituted flavonoids. The spectrum at 350 nm showed strong activity from flavonoid compounds with extended chromophore systems (flavones, flavonols). In comparison, at 400 nm, oxidized pigments and flavone derivatives associated with less polar structures were detected. Overall, the most complex and intense profiles appeared between 200 and 350 nm, a region specific to phenolic and flavonoid compounds. These findings support the notion that *Capsella bursa-pastoris* mainly contains polar secondary metabolites with high antioxidant activity, consistent with the existing literature data [13].

4. Conclusions

It can be concluded that *Trifolium pratense* is a valuable phytochemical source with greater therapeutic potential compared to *Capsella bursa-pastoris* due to its higher content of phenolic and isoflavone compounds. These may help protect against oxidative stress and skin tumor processes. The TLC method proved effective for the preliminary identification of bioactive compounds, allowing the phytochemical differences between the two species to be highlighted. The results support the development of characteristic chromatographic profiles and reinforce the hypothesis that red clover has superior potential as a natural source of compounds with anticarcinogenic effects on the skin. Densitometric analysis confirmed the presence of nine major compounds with strong UV absorption, primarily polyphenols and isoflavones, validating the chromatographic profile and emphasizing the chemical complexity of *T. pratense* extract. Overall, the findings underscore the importance of this species as a promising source of antioxidant and anticarcinogenic agents with potential applications in developing phytotherapeutic products for skin conditions. Based on this perspective, future research should focus on isolating and characterizing active compounds, evaluating antioxidant and antiproliferative biological mechanisms, and developing innovative topical formulations (creams, gels, serums) based on standardized *Trifolium pratense* extracts to validate their clinical efficacy and safety.

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Author contributions

Conceptualization, A.-M.C. and D.M.B.; methodology, A.-M.C.; formal analysis, D.G.; investigation, A.-M.C. and D.M.B.; resources, A.-M.C.; writing—original draft preparation, A.-M.C. and I.S.; writing—review and editing, D.G.; visualization, I.S.; supervision, D.M.B. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

Data supporting these findings are available within the article or upon request.

Institutional review board statement

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Informed consent statement

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