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Red Clover and the Importance of Extraction Processes—Ways in Which Extraction Techniques and Parameters Affect *Trifolium pratense* L. Extracts' Phytochemical Profile and Biological Activities

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Abstract: The purpose of this study was to gain an insight into the manner in which several extraction processes (both classical as well as innovative) affected bioactive compound yield, and subsequently to assess several of their biological activities. Red clover extracts were obtained using maceration, Soxhlet extraction, turbo-extraction, ultrasound-assisted extraction, and a combination of the last two. The resulting extracts were analyzed for total phenolic and flavonoid content. The extracts presenting the best results were subjected to a phytochemical assessment by way of HPLC-MS analysis. After a final sorting based on the phytochemical profiles of the extracts, the samples were assessed for their antimicrobial activity, anti-inflammatory activity, and oxidative stress reduction potential, using animal inflammation models. The Soxhlet extraction yielded the most satisfactory results both qualitatively and quantitatively. The ultrasound-assisted extraction offered comparable yields. The extracts showed a high potential against gram-negative bacteria and induced a modest antioxidant effect on the experimental inflammation model in Wistar rats.

Keywords: red clover; extraction methods; biological activity; HPLC; phytochemical profile; antimicrobial activity; oxidative stress reduction; anti-inflammatory activity; ultrasound-assisted extraction; innovative extraction methods

1. Introduction

Having been cultivated no sooner than the third century by European farmers, red clover is still to this day used in agriculture as a cover crop due to its weed suppression capacity, atmospheric nitrogen fixation, soil conservation, and multiple other advantages [1].

The genus *Trifolium* consists of hundreds of annual and perennial species, with short stems, trifoliate compound leaves, and sessile, outwardly spread flowers, as is the case for *Trifolium pratense* L. The corolla ranges in coloring from pink to red or even purple. Flowering periods range from the early months of spring to summer, and even early autumn [2,3].

Apart from its agricultural benefits, numerous applications for red clover have existed in medicine throughout history. The plant is mainly known for its isoflavonic compounds, such as formononetin, biochanin A, ononin, daidzein and genistein, which are considered as

phytoestrogens with estrogenic, anti-tumor, anti-inflammatory activity, as well as showing potential effects on cardiovascular risks, neuroprotective effects, and many others [4–10].

Conventional extraction methods have recently been regarded as more disadvantageous due to the necessity of large volumes of solvents, which are often polluting or toxic. These methods also involve many time-consuming steps. Maceration, decoction, infusion, Soxhlet extraction, etc. can be considered major examples of conventional extraction methods. Thus, unconventional extraction methods have come into focus by virtue of their advantages, e.g., decreased solvent volumes, with lower toxicity, reduced extraction time, and so on. Such examples of extraction methods imply the assistance of microwaves, ultrasounds, pressurized fluids, etc. [11,12].

Based on current findings, few reports concerning the influence of extraction methods over the phytochemical profile or biological activity of the species *Trifolium pratense* L. exist [13,14].

The main objective of this study was to evaluate the influence of extraction methods and parameters on the phytochemical profiles and biological activities of *Trifolium pratense* L. extracts. Furthermore, the intention was to offer a comparison between the results of the different extraction methods. The extraction methods that were taken into consideration were maceration, Soxhlet extraction, turbo-extraction, ultrasound-assisted extraction, and finally, a combination of the last two methods. Experimental Wistar rat plantar inflammation models induced by carrageenan administration were employed to elucidate anti-inflammatory and antioxidant effects. Antimicrobial activity was also assessed.

2. Materials and Methods

2.1. Plant Material

Dried aerial parts of *Trifolium pratense* L. were purchased from a local tea company (Hypericum Impex, Baia Sprie, Maramureș, Romania). The plant material was ground to a fine powder with an electric grinder.

2.2. Chemicals, Reagents, and Devices

Folin–Ciocâlteu reagent, sodium carbonate (Na_2CO_3), Aluminum chloride ($AlCl_3$), ABTS (diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate), DPPH (2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), indomethacin, carboxymethylcellulose, o-phthalaldehyde, Lambda carrageenan type IV, were purchased from Sigma–Aldrich (Taufkirchen, Germany). 2-thiobarbituric acid and Bradford reagent were acquired from Merck KGaA (Darmstadt, Germany) and ELISA cytokines tests (TNF-α and IL-6, respectively) were purocured from Elabscience (Houston, TX, USA). Bradford total protein assay was purchased from Biorad (Hercules, CA, USA). All analytical grade, HPLC reagents and standards were obtained from Sigma–Aldrich (Taufkirchen, Germany) and Decorias (Valea Lupului, Romania).

The following devices were used: Bosch MKM6003 grinder (Gerlingen, Germany), SER 148 solvent extraction unit (VELP® Scientifica, Usmate Velate, Italy), T 50 ULTRA-TURRAX® disperser (IKA®-Werke GmbH & Co. KG, Staufen, Germany), Sonic-3 ultrasonic bath (Polisonic, Warsaw, Poland), refrigerated high speed centrifuge Sigma 3–30 KS (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), Specord 200 Plus spectrophotometer (Analytik Jena, Jena, Germany), Agilent 1100 Series HPLC Value System coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap SL) (Agilent Technologies, Santa Clara, CA, USA), Bioblock Scientific 94,200 rotary evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), vacuum controller HS-0245 (Hahnshin Scientific Co., Tongjin-eup, Gimpo-si, Gyeonggi-do, Republic of Korea), Brinkman Polytron homogenizer (Kinematica AG, Littau-Luzern, Switzerland).

2.3. Selected Extraction Methods

The following extraction conditions were maintained constant for all the selected extraction techniques: the solvent was 70% alcohol and the solvent to sample ratio was

10:1 (*v*/*w*). This decision was made in order to ensure the correspondence of the results and to permit an accurate comparison between the selected extraction methods. After each completed extraction, separation was further carried out through centrifugation at 12,000 rpm for 10 min.

2.3.1. Maceration

Extraction was carried out according to the specifications of the Romanian Pharmacopoeia. Five grams of plant material were placed in a Falcon flask and 50 mL 70% alcohol were added. The contents of the flask were kept at room temperature for 10 days and mixed periodically. After the extraction was concluded, the sample was separated.

2.3.2. Soxhlet Extraction (SE)

In each extraction cup, 5 g plant material were added, followed by 50 mL 70% alcohol. For this extraction method, the heating plate was set to 210 °C in order to permit the solvent to reach its boiling point of approximately 79 °C. The duration of the extraction process was varied from 20 min to 40 min, and to 60 min, respectively. Separation followed the extraction process.

2.3.3. Turboextraction (TBE)

For each extract, 5 g plant material were added in a beaker, followed by 50 mL 70% alcohol. The parameters selected for study were extraction time and rotation speed. Extraction time was calculated in such a way as to represent 10 min and 20 min, respectively. For this, the extraction time of 10 min was divided into 2 extraction cycles, each of 5 min. In addition, the extraction time of 20 min was divided into 4 cycles, each of 5 min. Dispersion was carried out in the that manner in order to prevent device overheating and evaporation of the solvent. The rotation speed values that were selected for study were as follows: 4.000 rpm, 6.000 rpm, and 8.000 rpm. The extraction process was followed by separation.

2.3.4. Ultrasound-Assisted Extraction (UAE)

Frequency was maintained at a constant of 50 Hz, and power at a constant value of 230 V, respectively. The assessed parameters were time, with values ranging from 10, 20 to 30 min and temperature, with values varying from 30, 40 to 50 °C, respectively. The extracts were then subjected to separation.

2.3.5. Combination of Two Extraction Methods: UAE and TBE (UTE)

The ultrasonic bath was brought to 30 °C. The ULTRA-TURRAX® disperser speed was set to 4.000 rpm. Extraction time was kept to a minimum of one cycle of 5 min. These parameters were selected in order to maintain solvent evaporation to a minimum and to avoid device overheating.

2.4. Determination of Total Phenolic Content (TPC)

The Folin–Ciocâlteu method was selected to determine the total polyphenolic content (TPC). The experiment was carried out according to the specifications of Csepregi et al. with several modifications [15]. Consequently, in a microtube, 270 µL Folin–Ciocâlteu reagent were mixed with 60 µL plant extract, after which 270 µL Na_2CO_3 6% (*w*/*v*) were added. Following an incubation period of 30 min, in a dark environment, absorbances of the samples were measured at 765 nm, with the selected standard of gallic acid. The results were expressed as mg gallic acid equivalents per mL extract (GAE mg/mL).

2.5. Determination of Total Flavonoid Content (TFC)

Total flavonoid content was analyzed through a modified version of the method used by Pinacho et al. [16]. 400 µL solution containing $AlCl_3$ 20 mg/mL in 5% acetic acid in ethanol 3:1 (*v*/*v*) ratio were added to 200 µL plant extract. Absorbances were determined at

420 nm, with quercetin as standard. Subsequently, results were expressed as mM quercetin equivalents (QE mM).

2.6. Antioxidant Activity Evaluation

2.6.1. DPPH Radical Scavenging Activity

The DPPH assay was carried out according to Martins et al. with slight modifications [17]. 800 µL DPPH radical methanolic solution were added to 200 µL extract. The mixture was incubated for 30 min, in a medium deprived of light, at 40 °C temperature. Absorbances were measured at 517 nm, having Trolox reagent as standard. The results that followed were expressed as mg Trolox equivalents per mL extract (TE mg/mL).

2.6.2. $ABTS^+$ Scavenging Activity

The experiment was performed using a method provided by Erel [18]. 200 µL acetate buffer 0.4 M, pH 5.8 were added to 20 µL $ABTS^+$ in acetate buffer 30 mM, pH 3.6 with 12.5 µL extract added to the previous mixture. The absorbances of the samples were determined at 660 nm, using Trolox as standard. Results were expressed as mM Trolox equivalents (mM TE).

2.6.3. FRAP Assay

The experiment followed the technique used by Csepregi et al. [15], with several alterations. FRAP reagent was obtained by adding 25 mL acetate buffer (300 mM, pH 3.6) to 2.5 mL TPTZ solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL $FeCl_3$ (20 mM in water). The freshly prepared reagent was added to 30 µL plant extract. The mixture was left to incubate for 30 min, then absorbances were measured at 620 nm. Trolox reagent was used as standard. Results were expressed as mM Trolox equivalents (TE mM).

2.7. HPLC-MS Analysis

The apparatus used for phytochemical analysis was an Agilent 1100 HPLC Series system (Agilent Inc., Santa Clara CA, USA) equipped with binary pump, degasser, autosampler, column thermostat, and UV detector. An Agilent 1100 mass spectrometer (LC/MSD Ion Trap SL) was coupled with the HPLC system. For the separation of the phenolic compounds, a reverse-phase analytical column was selected (Zorbax SB-C18, 100 µm × 3.0 µm i.d., 3.5 µm particle size). The working temperature was set at 48 °C. Compounds' detection was performed on both MS mode and UV. The UV detector operated at different wavelengths (330 nm until 17.5 min, followed by 370 nm until the end of analysis). For the MS system, an electrospray ionization source operated in negative mode with a capillary potential of +3000 V, a nebulizer pressure of 60 psi (nitrogen), a flow rate for nitrogen gas of 12 L/min, and a dry gas temperature of 360 °C. The MS operated in monitoring specific ions mode (for polyphenol carboxylic acids) or AUTO Ms (for flavonoids and their aglycones). Separation of the phenolic compounds was carried out with a mobile phase of methanol:acetic acid 0.1% (v/v) and elution was in a binary gradient (at start, elution was with a linear gradient, from 5% to 42% methanol at 35 min, kept at isocratic elution for the following 3 min with 42% methanol, and then rebalance the column with 3% methanol). The flow rate was set at 1 mL/min and the injection volume was of 5 µL [19–21].

Another LC-MS analytical method was used to identify the following polyphenols in the vegetal extracts: epicatechin, catechin, syringic acid, gallic acid, protocatechuic acid, and vanillic acid. Chromatographic separation was carried out on a Zorbax SB-C18 column (100 mm × 3.0 mm i.d., 3.5 µm particle size) under a binary gradient and with a mixture of methanol:acetic acid 0.1% (v/v) as the mobile phase (3% methanol at start, 8% methanol at 3 min, 20% methanol from 8.5 to 10 min, followed by 3% methanol to rebalance the column). The flow rate was set at 1 mL/min, while the injection volume was of 5 µL. The MS mode (SIM-MS) was selected to detect the polyphenolic compounds. The MS system operated under the same conditions as described above [22,23].

Sterolic compounds separation was performed under isocratic elution conditions, with a Zorbax SB-C18 RP analytical column (100 mm × 3.0 mm i.d., 3.5 µm particle size). The mobile phase consisted of methanol:acetonitrile 10:90 (*v*/*v*). For MS analyses the positive ion mode was selected and was performed with the apparatus Agilent Ion Trap 1100 SL MS, with an APCI interface. The nitrogen gas temperature was set at 325 °C, with a flow rate of 7 L/min, a nebulizer pressure of 60 psi, and a capillary voltage of −4000 V. For identification of sterolic compounds, the MS spectra and RTs were compared with those obtained under the same conditions for standard compounds. The multiple reactions monitoring analysis mode (MS/MS) was selected to decrease the background interference. Under the described chromatographic conditions, the retention times of the four analyzed sterols were: 2.4 min for ergosterol, 3.7 min for both stigmasterol and campesterol (coelution), and 4.2 min for sitosterol [24,25].

Data Analysis (v5.3) and ChemStation (vA09.03) software from Agilent Inc. (Santa Clara, CA, USA) were used to obtain and analyze the chromatographic data.

2.8. Assessment of Antimicrobial Activity

2.8.1. In Vitro Qualitative Evaluation of Antimicrobial Activity

The antimicrobial potential was assessed by the disk diffusion test, using standard strains of Gram-positive and Gram-negative bacteria as well as yeasts. Four Gram-positive microbial strains were selected: *Staphylococcus aureus* ATCC 6538P, *Listeria monocytogenes* ATCC 13932, *Enterococcus faecalis* ATCC 29212, and *Bacillus cereus* ATCC 11778. Three Gram-negative strains were selected: *Escherichia coli* ATCC 10536, *Salmonella enteritidis* ATCC 13076 and *Pseudomonas aeruginosa* ATCC 27853. The selected yeast strain was *Candida albicans* ATCC 10231. The selected standard antibacterial control was amoxicillin, whereas ketoconazole was selected as a yeast standard control.

Screening was carried out according to EUCAST standards [26]. A suspension was prepared from young microbial colonies (24 h old), grown on Mueller-Hinton (MH) agar for bacteria, and Sabouraud dextrose agar (SDA). It was further adjusted at 0.5 density in saline on McFarland scale using Densichek calibration standard (bioMérieux, Craponne, France). 8.5 cm diameter plastic Petri dishes with MH agar for bacteria and SDA agar for yeast were flooded with the resulted suspension. Once the excess fluid was eliminated, the agar surface was left to dry, and 5 mm diameter filter paper discs were placed in a radial model. 20 µL were added on each filter paper disk and the plates were incubated for 18 h at 35 ± 2 °C for bacteria and 48 h at 28 °C for the fungal strain [26]. The antimicrobial activity was measured by determining the diameter of the growth inhibition area, with results expressed in mm.

2.8.2. In Vitro Quantitative Evaluation of Antimicrobial Activity

The antimicrobial activity was quantitatively measured following the minimum inhibitory concentration (MIC) method for the eight above-mentioned microbial strains. The assessment was accomplished by modified EUCAST protocols [26]. 96-wells titer plates were used. The extracts were added, once diluted in liquid MH medium, and afterwards inoculated with 20 µL microbial suspension. The stock solutions of the extracts were diluted using a two-fold serial dilution system in ten consecutive wells, from the initial concentration (1/1) to the highest (1/512). The total broth volume was adjusted to 200 µL. Positive controls represented by microbial inoculum in MH broth and negative control represented by microbial inoculum in 30% ethanol were also prepared and used to fill wells 11 and 12, respectively. The incubation period of the wells was 24 h at a temperature of 37 °C for bacteria and 48 h at 28 °C for *Candida*, respectively. MIC values were determined as the lowest concentration of the extracts' dilution that inhibited the growth of the microbial cultures (having the same OD as the negative control), compared to the positive control, as established by a decreased value of absorbance at 450 nm (HiPo MPP-96, Biosan, Latvia). MIC50 was also determined, representing the MIC value at which ≥50% of the

bacterial/yeast cell growth was inhibited, considered as the well with the OD value similar to the average between the positive and negative control.

2.9. Determination of Biological Activities

Once the phytochemical profile of the extracts was determined, the 60 min SE was selected for further determination of in vivo biological activities. The selection was based on number of identified compounds, and concentration levels of said compounds.

2.9.1. Inflammation Model in Rats Induced by Carrageenan

The in vivo determination was accomplished by way of an inflammation experimental model in male Wistar rats (110–130 g mean weight). The specimens were acclimatized in the ensuing conditions: 12 h light/12 h dark cycles, 35% humidity, free access to water, and a normocaloric standard diet (VRF1), randomization in 4 groups, 8 specimens each. During a period of 4 days, treatment was administered by oral gavage, in a maximum volume of 0.25 mL, such as: group 1—carboxymethylcellulose 2% (positive control group—CMC); group 2—Indomethacin 5 mg/body weight (b.w.) in carboxymethylcellulose 1.5% (Indom); group 3–20 mg TPC/b.w./day (60 min SE).

On the fifth day, inflammation was induced by injecting 100 µL of freshly prepared 1% carrageenan (λ-carrageenan, type IV, Sigma) diluted in normal saline in the right hind footpad (32). An exact saline solution volume was injected at the level of the left hind paw, serving as negative control. Subsequently, at 2 and 24 h after carrageenan administration, soft paw samples were collected under intraperitoneally injected 90 mg/kg ketamine and 10 mg/kg xylazine. The samples were assessed for oxidative stress parameters and cytokine levels, following homogenization in 50 mMTRIS–10 mM EDTA buffer (pH 7.4) [27]. The protein content was evaluated using the Bradford method [28].

The experiments were permitted by the Ethic Committee Board of "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania (291/23.02.2022) on Animal Welfare according to the Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.9.2. Evaluation of Oxidative Stress

The levels of malondialdehyde (MDA), glutathione reduced and glutathione oxidized levels and GSH/GSSG ratio were determined in the from paw tissue homogenates. Spectrofluorimetry was used to quantify MDA levels, by 2-thiobarbituric acid method [29]. The GSH and GSSG levels were determined by the Hu method [30].

2.9.3. Evaluation of Proinflammatory Cytokines

TNF-α and IL-6 levels from plantar tissue homogenates were evaluated by ELISA assay following the protocol provided by the manufacturer. Results were expressed in pg/mg protein.

2.9.4. Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyze data, followed by the Tukey's multiple comparisons post-test, using GraphPad Prism 8 software. A p value < 0.05 was considered statistically significant. The results were expressed as mean ± standard deviation.

3. Results

A final number of 20 extracts was reached after each extraction process was finished. One extract was performed by maceration (M), three samples were obtained through SE (S), six samples by turboextraction (T), nine samples were prepared through UAE (U) and the last remaining sample was completed by UTE (UT). The initials used to label the samples represent the abbreviation of the extraction method and the parameters that were studied for each item: M for maceration, S for SE, U for ultrasound, and UT for UTE. Table 1

represents the explained nomenclature of the extract samples which were evaluated in this study.

Table 1. Nomenclature of the obtained extract samples of the study.

Extraction Method	Studied Extraction Parameters			Sample Name
Maceration	*			M
Soxhlet extraction (SE)	Time (min)	20		S20
		40		S40
		60		S60
Turboextraction (TBE)		10 min (2 cycles of 5 min)	Rotation peed (rpm)	4.000 T24
				6.000 T26
				8.000 T28
		20 min (4 cycles of 5 min)		4.000 T44
				6.000 T46
				8.000 T48
Ultrasound-assisted extraction (UAE)		10	Temperature (°C)	30 U13
				40 U14
				50 U15
		20		30 U23
				40 U24
				50 U25
		30		30 U33
				40 U34
				50 U35
Combination of two extraction methods: UAE and TBE (UTE)	**			UT

* Parameters remained constant, see Section 2.3.1. Maceration, ** Parameters remained constant, see Section 2.3.5. Combination of two extraction methods: UAE and TBE (UTE).

Finally, the extracts that offered the highest yields were selected for further HPLC analysis. After the phytochemical characterization of the respective extracts, the biological activity of the samples presenting the highest concentration of antitumoral compounds was evaluated.

3.1. Effect of Extraction Parameters on TPC and TFC Values

Table 2 contains the TPC and TFC values of the studied extracts.

Table 2. TPC and TFC of the extracts.

Sample	TPC (GAE mg/mL) *	TFC (QE mM) *
M	0.970 ± 0.022	1.216 ± 0.046
S20	1.146 ± 0.010	1.876 ± 0.019
S40	1.186 ± 0.013	1.331 ± 0.045
S60	1.223 ± 0.017	2.276 ± 0.009
T24	0.818 ± 0.020	1.465 ± 0.039
T26	0.624 ± 0.011	1.320 ± 0.004
T28	0.636 ± 0.014	0.930 ± 0.040
T44	0.708 ± 0.006	1.262 ± 0.024
T46	0.714 ± 0.016	1.070 ± 0.018
T48	0.619 ± 0.017	1.143 ± 0.009
U13	0.522 ± 0.009	0.770 ± 0.041
U14	0.823 ± 0.007	1.303 ± 0.008
U15	0.560 ± 0.017	1.148 ± 0.028
U23	0.728 ± 0.000	0.956 ± 0.028
U24	0.558 ± 0.004	0.970 ± 0.047
U25	0.747 ± 0.015	1.863 ± 0.047
U33	0.673 ± 0.019	1.854 ± 0.034
U34	0.610 ± 0.023	2.161 ± 0.043
U35	0.838 ± 0.030	1.414 ± 0.076
UT	0.687 ± 0.017	0.694 ± 0.038

* concentrations were expressed as mean ± SD.

SE provided the extracts with the highest TPC values. The increase of extraction time was inversely proportional to the polyphenolic content. Thus, in the case of SE, the extraction period of 60 min proved to be the optimal extraction time for this particular plant material and solvent. Maceration followed, having achieved a lower value than SE. Turbo-extraction was comparable to the UAE, although the values resulted from these extraction methods were greatly inferior to the previously mentioned methods. For TBE, increasing extraction time (from two cycles of 5 min to four cycles of 5 min) and speed, particularly from 6.000 rpm to 8.000 rpm, both determined lower yields. The highest TPC value was reached with the parameters of two cycles of 5 min as extraction period and the lowest speed value, 4.000 rpm, respectively. Highest values of TPC for UAE were achieved when the parameter combinations of 10 min extraction time with 40 °C, and 30 min extraction time with 50 °C were used. The combination of UAE and TBE did not reach a comparable value with either SE or maceration.

For TFC, even though SE reached the highest values in this case as well, UAE gave comparable results with SE. Maceration showed a medium value. The lowest value was attained by UTE. Prolonged extraction time, i.e., 60 min for SE enabled the highest yield. This was followed by the UAE extracts, with optimal extraction conditions having been 30 min extraction time and 40 °C. Other UAE conditions comparable with these were the combinations of 20 min with 50 °C and 30 min with 30 °C.

3.2. Effect of Extraction Parameters on Antioxidant Activity

Results are detailed in Table 3.

Table 3. Antioxidant capacity of the extracts.

Sample	DPPH (TE mg/mL) *	FRAP (TE mM) *	$ABTS^+$ (TE mM) *
M	2.721 ± 0.445	5.465 ± 0.196	2.97 ± 1.121
S20	2.541 ± 0.049	8.444 ± 0.165	4.687 ± 0.389
S40	2.718 ± 0.132	8.476 ± 0.365	3.525 ± 0.810
S60	2.822 ± 0.067	8.615 ± 0.326	5.646 ± 0.457
T24	3.740 ± 0.337	8.615 ± 0.217	3.551 ± 0.417
T26	4.430 ± 0.233	9.564 ± 0.405	3.626 ± 0.191
T28	2.270 ± 0.143	8.172 ± 0.027	3.576 ± 0.330
T44	2.894 ± 0.100	8.836 ± 0.270	4.131 ± 0.381
T46	2.549 ± 0.144	9.564 ± 0.377	3.778 ± 0.287
T48	2.801 ± 0.183	9.231 ± 0.190	2.843 ± 0.116
U13	2.271 ± 0.017	3.808 ± 0.027	2.894 ± 0.131
U14	3.452 ± 0.031	9.374 ± 0.142	4.535 ± 0.747
U15	2.241 ± 0.025	1.262 ± 0.047	3.475 ± 0.044
U23	2.231 ± 0.008	6.447 ± 0.123	4.182 ± 0.473
U24	1.648 ± 0.022	5.047 ± 0.179	3.475 ± 0.087
U25	3.345 ± 0.022	10.923 ± 0.152	5.04 ± 0.231
U33	2.802 ± 0.059	7.097 ± 0.207	4.485 ± 0.227
U34	2.434 ± 0.031	5.405 ± 0.072	4.081 ± 0.087
U35	2.444 ± 0.039	7.255 ± 0.198	5.167 ± 0.076
UT	3.253 ± 0.029	6.512 ± 0.152	4.056 ± 0.044

* concentrations were expressed as mean ± SD.

A prime example appeared for the DPPH assay, where the highest value was obtained by TBE, at the shorter extraction time used, i.e., two cycles of 5 min and a medium speed value, i.e., 6.000 rpm. This was followed by a similar combination of parameters, with the exception of the speed value being 4.000 rpm.

FRAP assay results were generally high for multiple methods, i.e., SE and TBE. Medium to low values were achieved for UAE overall. Nevertheless, the highest value of TE was attained by UAE, at 20 min time with 50 °C, respectively.

For the ABTS+ assay however, the highest values were observed for SE, with the longest extraction time of 60 min having been the optimal value in this case. This was

followed by UAE, with the parameters most favorable consisting of 20 min extraction time and 50 °C as well as 30 min and 50 °C, respectively.

3.3. HPLC-MS Analysis of the Extracts

Out of the initial 20 extracts, 11 were selected for further HPLC-MS analysis. The selected extracts presented the highest values of the results obtained for the assays carried out as mentioned above, in Section 3.1. and Section 3.2.

3.3.1. Evaluation of Polyphenolic Compounds

Table 4 details the results following the HPLC-MS detection and quantification of polyphenolic compounds in the studied extracts.

Table 4. Polyphenolic compounds in the selected extracts.

Sample	Catechin (µg/mL Extract) *	Syringic Acid (µg/mL Extract) *	Protocatechuic Acid (µg/mL Extract) *	Vanillic Acid (µg/mL Extract) *	Chlorogenic Acid (µg/mL Extract) *	*p*-Coumaric Acid (µg/mL Extract) *	Caftaric Acid (µg/mL Extract) *
M	ND	0.13 ± 0.004	1.31 ± 0.145	0.45 ± 0.022	3.57 ± 0.393	1.83 ± 0.201	ND
S40	ND	0.16 ± 0.013	0.88 ± 0.124	0.37 ± 0.026	8.78 ± 0.263	1.89 ± 0.283	ND
S60	ND	0.13 ± 0.005	1.11 ± 0.089	0.38 ± 0.042	9.76 ± 0.293	2.49 ± 0.174	ND
T24	ND	0.10 ± 0.011	0.55 ± 0.039	0.23 ± 0.028	5.61 ± 0.393	1.34 ± 0.040	ND
T26	0.05 ± 0.004	0.06 ± 0.004	0.39 ± 0.027	0.17 ± 0.017	5.46 ± 0.164	1.10 ± 0.113	ND
T46	0.08 ± 0.010	0.09 ± 0.003	0.48 ± 0.033	0.27 ± 0.008	6.97 ± 0.836	1.40 ± 0.211	4.51 ± 0.541
U14	0.02 ± 0.003	0.08 ± 0.005	0.56 ± 0.040	0.30 ± 0.024	6.04 ± 0.544	1.65 ± 0.181	ND
U25	ND	0.14 ± 0.012	0.78 ± 0.086	0.31 ± 0.016	5.91 ± 0.296	1.77 ± 0.159	ND
U34	0.05 ± 0.003	0.08 ± 0.009	0.49 ± 0.063	0.19 ± 0.013	4.78 ± 0.478	1.40 ± 0.098	ND
U35	0.11 ± 0.016	0.09 ± 0.013	0.81 ± 0.122	0.32 ± 0.044	7.73 ± 0.618	2.01 ± 0.281	ND
UT	0.04 ± 0.005	0.09 ± 0.014	0.75 ± 0.752	0.39 ± 0.043	8.10 ± 0.810	1.89 ± 0.245	ND

* concentrations were expressed as mean ± SD.

Low levels of catechin were identified. Only TBE, UAE and UTE extracts contained this compound. Seemingly, the increase in extraction time, speed or temperature enabled the extraction of catechin, i.e., four cycles of 5 min and 6.000 rpm for TBE and 30 min and 50 °C for UAE. However, UAE reached the highest yield.

Other polyphenolic compounds such as syringic acid, protocatechuic acid, vanillic acid, chlorogenic acid, and *p*-coumaric acid were detected in all analyzed samples. The highest levels of syringic acid were found in the SE sample that was subjected to 40 min extraction time, with an increase in temperature showing a decrease in concentration. Other notable extraction methods and conditions that enabled high yield levels for syringic acid were UAE (20 min, 50 °C) and maceration. Protocatechuic acid reached the highest level in the macerate, followed by the SE extract with 60 min extraction time. Vanillic acid was also detected in high concentrations in the macerate, followed by the combination of TBE and UAE, and SE, respectively. The chlorogenic and *p*-coumaric acids were both detected in high concentrations in the SE extracts that were subjected to a 60 min process time.

Interestingly, the caftaric acid was detected only in one sample, namely the extract obtained through TBE, at four cycles of 5 min and speed of 6.000 rpm, respectively.

3.3.2. Evaluation of Flavonoid Compounds

Results are illustrated in Table 5.

A total of 11 flavonoid compounds were identified in the studied extracts. With eupatilin having been identified in strictly one of the samples. These findings were in accordance with previous reports in scientific literature affirming the wide variety of flavonoid and isoflavonoid compounds in the species pertaining to the genus *Trifolium* [2,3,31,32].

The highest concentrations were reached throughout all extracts for the compounds jaceosidin and hispidulin. These were followed by hyperoside, isoquercitrin and quercitrin, respectively. Generally, SE at 60 min extraction time enabled the highest yields. The exception of rutin, where TBE led to the highest yield, and eupatilin that was identified

solely in a UAE extract (30 min extraction time and 40 °C temperature). Overall, low to medium values were detected in samples obtained by maceration, TBE and UAE. However, in the case of rutin, the sample subjected to TBE at four cycles of 5 min and 6.000 rpm enabled a large yield.

Table 5. Flavonoid compounds in the selected extracts.

Sample	Kaempferol (µg/mL Extract) *	Quercetol (µg/mL Extract) *	Isoquercitrin (µg/mL Extract) *	Quercitrin (µg/mL Extract) *	Rutin (µg/mL Extract) *	Hyperoside (µg/mL Extract) *	Luteolin (µg/mL Extract) *	Vitexin (µg/mL Extract) *	Apigenin (µg/mL Extract) *	Eupatilin (ng/mL Extract) *	Jaceosidin (ng/mL Extract) *	Hispidulin (ng/mL Extract) *
M	ND	1.72 ± 0.069	ND	ND	ND	ND	2.27 ± 0.113	1.77 ± 0.247	0.78 ± 0.078	ND	249.29 ± 19.943	371.04 ± 18.552
S40	0.35 ± 0.011	6.95 ± 0.208	2.05 ± 0.061	17.19 ± 1.547	ND	ND	2.27 ± 0.249	2.85 ± 0.285	0.68 ± 0.081	ND	295.57 ± 17.734	343.90 ± 37.829
S60	ND	8.93 ± 0.268	1.58 ± 0.047	7.47 ± 0.672	ND	ND	3.03 ± 0.393	3.26 ± 0.359	1.07 ± 0.128	ND	408.32 ± 48.999	453.76 ± 36.301
T24	ND	4.80 ± 0.144	1.28 ± 0.128	7.66 ± 1.072	ND	ND	1.71 ± 0.086	1.50 ± 0.045	0.68 ± 0.082	ND	199.96 ± 13.997	280.16 ± 16.810
T26	ND	4.80 ± 0.240	0.97 ± 0.087	7.66 ± 0.230	ND	ND	1.51 ± 0.211	1.50 ± 0.209	0.58 ± 0.058	ND	188.79 ± 11.328	260.63 ± 10.425
T46	ND	4.58 ± 0.412	0.66 ± 0.066	4.85 ± 0.146	ND	ND	1.78 ± 0.160	2.58 ± 0.129	0.68 ± 0.088	ND	232.15 ± 20.894	313.70 ± 47.055
U14	0.28 ± 0.014	4.52 ± 0.633	2.35 ± 0.330	10.09 ± 1.513	ND	ND	1.85 ± 0.259	2.17 ± 0.217	0.58 ± 0.081	ND	261.53 ± 28.768	272.18 ± 19.053
U25	0.28 ± 0.020	4.58 ± 0.595	3.59 ± 0.323	12.33 ± 0.740	15.55 ± 1.711	ND	1.99 ± 0.258	2.31 ± 0.277	0.68 ± 0.061	ND	300.05 ± 18.003	300.51 ± 21.036
U34	0.42 ± 0.012	4.03 ± 0.443	3.28 ± 0.492	15.14 ± 0.454	ND	ND	1.51 ± 0.135	1.90 ± 0.133	0.58 ± 0.075	4.00 ± 0.360	224.71 ± 26.966	228.09 ± 6.843
U35	0.55 ± 0.082	5.90 ± 0.413	4.05 ± 0.445	20.56 ± 0.617	ND	ND	2.20 ± 0.066	2.58 ± 0.155	0.78 ± 0.047	ND	301.81 ± 39.235	375.38 ± 15.015
UT	0.42 ± 0.058	5.02 ± 0.452	3.28 ± 0.361	15.14 ± 0.605	ND	1.86 ± 0.167	2.06 ± 0.288	2.45 ± 0.294	0.78 ± 0.023	ND	295.73 ± 17.744	328.26 ± 9.848

* concentrations were expressed as mean ± SD.

3.3.3. Evaluation of Isoflavonoid Compounds

Results of the evaluation of the isoflavonoid compounds are detailed in Table 6.

Table 6. Isoflavonoid compounds in the selected extracts.

Sample	Daidzein (ng/mL Extract) *	Daidzin (ng/mL Extract) *	Genistein (ng/mL Extract) *	Genistin (ng/mL Extract) *	Glycitein (ng/mL Extract) *
M	483.11 ± 53.142	102.14 ± 14.299	388.73 ± 19.437	973.82 ± 48.691	ND
S40	361.67 ± 36.167	161.11 ± 8.055	337.51 ± 50.626	1524.99 ± 76.249	ND
S60	528.48 ± 15.854	235.53 ± 23.553	454.45 ± 59.079	1942.73 ± 58.282	33.11 ± 4.304
T24	367.83 ± 40.461	140.07 ± 9.805	295.98 ± 11.839	944.15 ± 75.532	ND
T26	277.34 ± 8.320	111.00 ± 3.330	240.41 ± 31.253	849.74 ± 101.969	ND
T46	371.48 ± 48.293	147.41 ± 19.164	352.37 ± 31.714	1450.72 ± 174.086	ND
U14	397.66 ± 55.673	223.70 ± 15.659	410.61 ± 49.273	1722.62 ± 241.167	16.24 ± 0.812
U25	431.24 ± 25.874	205.86 ± 10.293	416.65 ± 54.164	1686.72 ± 118.070	35.60 ± 2.136
U34	377.06 ± 37.706	139.86 ± 8.394	341.87 ± 51.280	1374.52 ± 68.726	ND
U35	483.47 ± 72.520	199.96 ± 9.998	443.84 ± 57.699	1714.08 ± 154.267	12.36 ± 1.112
UT	423.72 ± 25.423	177.90 ± 8.895	434.44 ± 26.066	1702.13 ± 85.107	20.84 ± 1.042

* concentrations were expressed as mean ± SD.

Five compounds, daidzein, daidzin, genistein, genistin, and glycitein were detected in the samples, in large concentrations. For these compounds, SE proved to be the most successful extraction method, with the extraction time of 60 min having been the most favorable time parameter, as well. Glycitein was detected only in certain samples, for instance, in the 60 min SE extract, three of the four UAE analyzed extracts and the UTE sample. UAE at 20 min time and 50 °C led to the highest yield in the case of this compound.

3.3.4. Evaluation of Sterolic Compounds

Results discussed in this section are found in Table 7.

Three sterolic compounds were detected in high levels in the samples: stigmasterol, β-sitosterol, and campesterol. For these compounds, UAE proved the most efficient extraction method, with maximum compound levels being extracted at 20 min, 50 °C for campesterol and 30 min, 50 °C for stigmasterol and β-sitosterol.

Table 7. Sterolic compounds in the selected extracts.

Sample	α-Tocopherol (ng/mL Extract)	γ-Tocopherol (ng/mL Extract)	Ergosterol (ng/mL Extract)	Stigmasterol (ng/mL Extract) *	β-Sitosterol (ng/mL Extract) *	Campesterol (ng/mL Extract) *
M	ND	ND	ND	2492.70 ± 99.708	62,032.15 ± 8684.501	1061.37 ± 127.364
S40	ND	ND	ND	1640.04 ± 246.007	34,113.14 ± 3070.183	498.55 ± 24.928
S60	ND	ND	ND	2609.65 ± 339.254	54,007.75 ± 6480.930	1100.60 ± 55.030
T24	ND	ND	ND	2356.91 ± 94.276	50,607.54 ± 5060.754	982.68 ± 29.480
T26	ND	ND	ND	2259.78 ± 293.771	42,076.96 ± 6311.544	710.98 ± 92.427
T46	ND	ND	ND	2370.90 ± 331.926	43,673.45 ± 3493.876	922.40 ± 27.672
U14	ND	ND	ND	3134.05 ± 282.065	56,018.75 ± 2800.938	1006.33 ± 140.887
U25	ND	ND	ND	3202.09 ± 320.210	68,060.97 ± 7486.707	1322.76 ± 52.911
U34	ND	ND	ND	2471.12 ± 197.690	52,752.50 ± 5802.775	1058.24 ± 148.154
U35	ND	ND	ND	3596.66 ± 323.700	83,130.15 ± 10,806.920	1277.69 ± 76.662
UT	ND	ND	ND	2955.13 ± 295.513	63,322.55 ± 5065.804	1014.49 ± 40.579

* concentrations were expressed as mean ± SD.

3.4. Evaluation of Antimicrobial Activity

3.4.1. In Vitro Qualitative Evaluation of Antimicrobial Activity

The potential of microbial growth inhibition of the extracts was screened by the disk diffusion test. The extracts showed high efficiency against Gram-negative bacteria, a moderate level against gram-positive bacteria and *Candida albicans*. Results are shown in Table 8.

Table 8. Results for the risk diffusion test performed for selected samples.

	U35 *	S60 *	Amoxicillin	Ketoconazole
Staphylococcus aureus ATCC 6538P	9.28	9.01	24.38	-
Enterococcus faecalis ATCC 29212	9.51	9.51	16.8	-
Listeria monocytogenes ATCC 13932	9.44	9.44	18.96	-
Bacillus cereus ATCC 11778	8.82	8.82	8.83	-
E. coli ATCC 10536	12.59	12.31	13.72	-
Salmonella enteritidis ATCC 13076	12.73	11.47	18.43	-
Pseudomonas aeruginosa ATCC 27853	11.27	10.3	R	-
Candida albicans 10231	9.11	8.83	-	23.74

* inhibition area diameter in mm; R—resistant.

The diameters of the inhibition areas were: 8.82–9.51 mm in the case of gram-positive strains, 10.3–12.73 mm regarding the gram-negative strains, and 8.83–9.11 mm for Candida albicans. Increased antimicrobial potential was demonstrated against gram-negative bacteria.

3.4.2. In Vitro Quantitative Evaluation of Antimicrobial Activity

Although the antimicrobial screening revealed high potential against gram-negative strains, the MIC method was used to evaluate the quantitative antimicrobial potential of the selected samples against all microbial strains that were used initially, in the qualitative part of the evaluation.

As presented in Table 9, lower MICs could be noted for gram-positive strains in this case, with an overall variation of the antimicrobial potential. A possible reason could be explained by a limited diffusion on the agar surface. A general high inhibitory concentration was observed for the wells with liquid MH medium.

Table 9. Results for the MIC test performed for selected samples.

	U35		S60	
	MIC 100	MIC 50	MIC 100	MIC 50
Staphylococcus aureus ATCC 6538P	1/16	1/32	1/32	1/32
Enterococcus faecalis ATCC 29212	1/64	1/64	1/64	1/64
Listeria monocytogenes ATCC 13932	1/32	1/64	1/32	1/64

Table 9. *Cont.*

	U35		S60	
	MIC 100	**MIC 50**	**MIC 100**	**MIC 50**
Bacillus cereus ATCC 11778	1/32	1/32	1/32	1/64
E. coli ATCC 10536	1/16	1/32	1/16	1/32
Salmonella enteritidis ATCC 13076	1/8	1/16	1/8	1/16
Pseudomonas aeruginosa ATCC 27853	1/16	1/16	1/16	1/32
Candida albicans 10231	1/32	1/32	1/32	1/32

3.5. Evaluation of Oxidative Stress and Inflammation Markers

Oxidative stress was evaluated by determination of lipid peroxidation marker, MDA, and by non-endogenous antioxidants levels such as reduced glutathione (GSH), oxidated glutathione (GSSG), and their respective ratio (GSH/GSSG). The activity of enzymatic antioxidants was also assessed: catalase (CAT) and glutathione peroxidase (GPx). Results are shown in Figure 1.

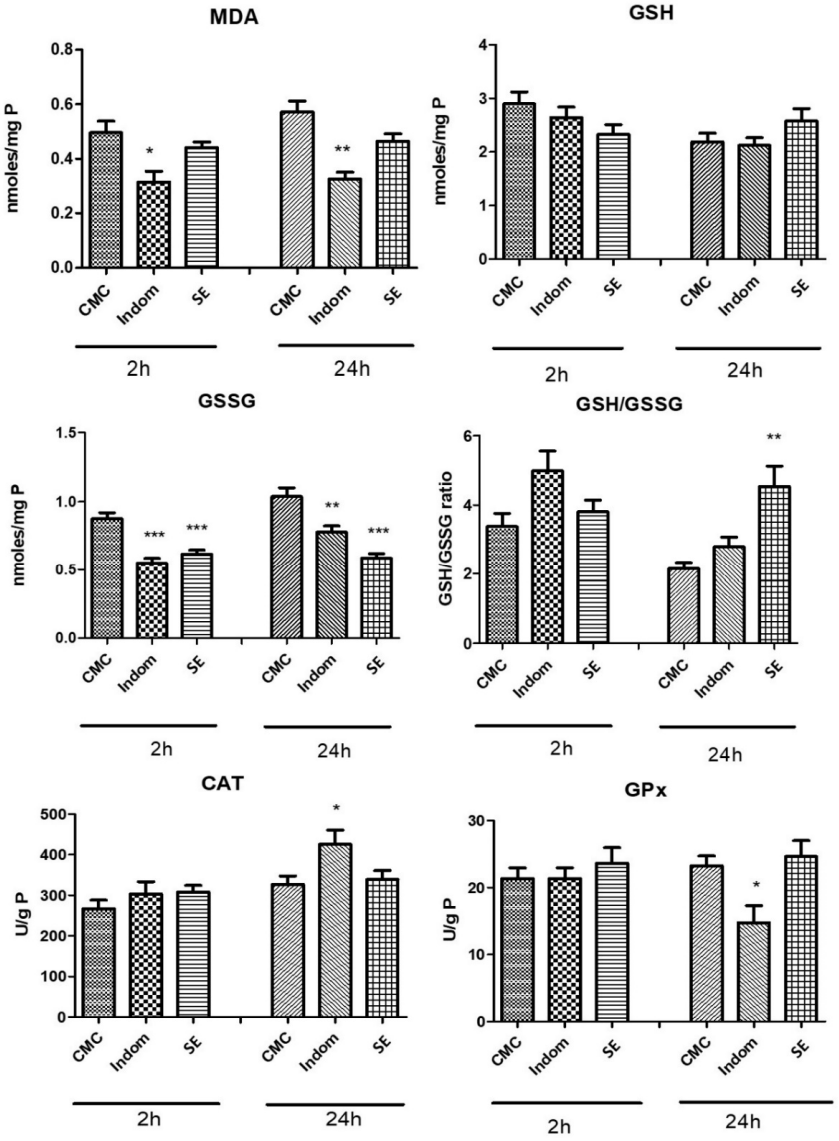

Figure 1. Soft paw tissue levels of MDA, GSG/GSSG ratio and the activity of CAT and GPx at 2, 24 h after carrageenan injection, in rats pretreated with SE extract. Values are means ± SD. Statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons post-test (* $p < 0.05$ ** $p < 0.001$ and *** $p < 0.0001$, all treated groups vs. control (CMC) group).

MDA levels decreased significantly only after Indom administration, both at 2 h ($p < 0.05$) and 24 h ($p < 0.01$) while the SE extract did not influence the lipid peroxidation ($p > 0.05$). SE positively influenced the GSSG levels, both at 2 h ($p < 0.001$) and 24 h ($p < 0.001$) after the carrageenan injection, similar to indomethacin and increased the GSH/GSSG ratio at 24 h ($p < 0.01$). CAT activity increased significantly after indomethacin administration at 24 h ($p < 0.05$) while the GPx activity decreased at 24 h in the same group ($p < 0.05$). The TP extract did not influence the antioxidant enzymes activity in paw tissue ($p > 0.05$).

Cytokine levels, such as IL-6 and TNF-α, were assessed in the plantar tissue homogenates compared with indomethacin, at 2 and 24 h after carrageenan injection. Results are illustrated in Figure 2. Compared to Indomethacin, SE administration did not significantly reduce the IL-6 and TNF-α secretions at 2 h and 24 h after induction of inflammation. Only Indomethacin administration decreased the TNF-α levels in the soft tissues at 24 h after carrageenan injection ($p < 0.05$).

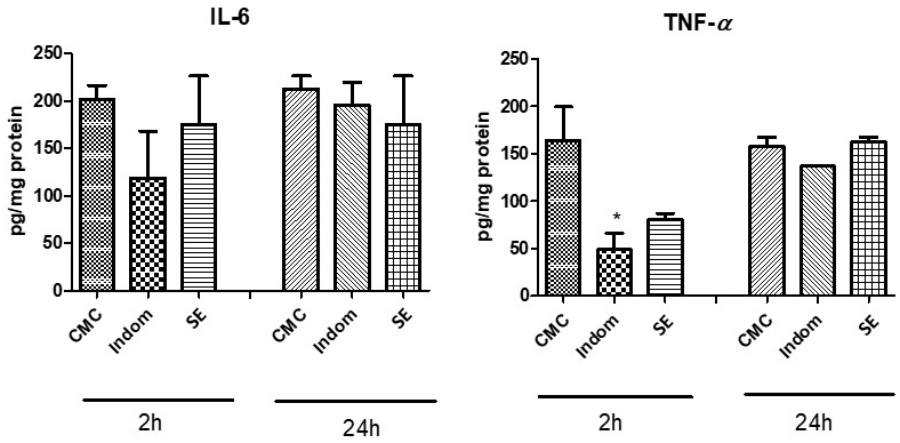

Figure 2. Levels of IL-6 and TNF-α in paw tissue of experimental rats at 2 h and 24 h after injection of carrageenan. Values are means ± SD. Statistical analysis was performed by a one-way ANOVA, with Tukey's multiple comparisons post-test (* $p < 0.05$ vs. control, CMC, group).

4. Discussion

According to the information available hitherto this study, few scientific reports were recently made concerning the influence of extraction methods or extraction parameters over the phytochemical profile or even biological activity of *Trifolium pratense* L. Two such examples, were focused on compounds belonging to the class of polysaccharides, the first consisting of an optimization of hot water extraction, with the second study following the effect of several extraction methods on these compounds [13,14]. Mikhailov et al. studied the influence of growth site on bioactive compounds, for samples of *T. pratense* L. and *T. repens* L. [33]. Vlaisavljević et al. have also studied the influence of growth phase on phenolic content on *T. pratense* L. extracts, obtained by means of a microwave-assisted extraction [34]. In 2009, two studies using micropreparative techniques, by Zgórka G., attempted to elucidate the effect of extraction methods and the respective parameters over the chemotaxonomy of isoflavonoid compounds from *Trifolium* species [35,36]. Visnevschi-Necrasov et al. have optimized an extraction method based on matrix solid-phase dispersion for isoflavonoid compounds from *T. pratense* L. [37].

Based on the obtained TPC results obtained in this study, it could be presumed that the subjection of the solvent to a higher temperature and prolonged extraction time, enabled greater degradation of cellular walls. This led to a larger release of polyphenolic content. As opposed to the TPC and TFC results, several differences were noticeable, with regards to the antioxidant activity of the extracts. This suggests that this method enabled the extraction of compounds with antioxidant capacity, contrary to the other implemented methods. Other noteworthy differences were represented by the UAE and UTE. In those cases, the parameters that permitted the achievement of higher TE values were 10 min extraction time with the temperature of 40 °C and 20 min extraction time at 50 °C for UAE,

and 30 °C with 4.000 rpm and 5 min extraction period for UTE. A possible explanation as to the reason why SE gave medium results might have been the degradation of said antioxidant compounds at high temperature values. Additionally, the low or medium parameters selected for TBE, and UAE showed similar results, in this case, antioxidant compound extraction having been possibly insufficient. The differences between values for each type of assay might reside in the particularity of each of these experiments: type of reagent used, required incubation time, or lack thereof, necessary ambient temperature, and so on.

Supposedly, UTE obtained a somewhat medium value due to the combination of all three parameters (time, temperature, speed) although values for these parameters were kept low due to the possible implications detailed in Section 2.3.5. Akbaribazm et al. have reported similar results, using a liquid chromatography-electrospray ionization mass spectrometry analysis method for hydroalcoholic extracts [32]. These findings were contrary to the results reported by Tundis et al. in which case rutin and quercitrin exhibited the highest values [31].

For isoflavonoid compounds, the findings were in accordance with previous scientific reports not only for the *T. pratense* L. species, but also concerning other members of this genus [32,38,39].

Despite newly developed industrial antibiotics, the resistance of microorganisms to these substances has grown. Generally, bacteria possess the genetical capacity to transmit and gain resistance towards therapeutic compounds. This fact becomes worrisome due to the increase of in-hospital patients which are immunosuppressed, but also due to the new multi-resistant strains. For this reason, new bacterial infections may arise, resulting in the increase of infectious pathologies. Such medical issues, derived from bacterial resistance, have found to be increasing. Thus, the use of antibacterial compounds has become troublesome. In this manner, healthcare specialists face the need of taking measures in order to reduce bacterial multi-resistance, by monitoring antibiotic use. Thus, the improvement of antibiotic treatment is desired. For a long period of time, plants have been a valuable resource of natural compounds used to maintain human health, and to this day, are still considered a possible alternative treatment, by way of natural therapies. For this reason, the pharmaceutical use of plant bioactive compounds has become a frontrunner. Current research suggests the effectiveness of plant bioactive compounds in treating infectious diseases. Therefore, plant-derived products can be considered promising, being used as antimicrobial agents. Plant-derived compounds also lack secondary effects, are easily available, and are low-cost [40].

Regarding the results of the antimicrobial activity of the presently studied extracts, the samples were found to present a high potential against gram-negative strains high potential against gram-negative strains. However, Khan et al. reported that for the extracts of the species *Trifolium alexandrium* L., the studied extracts presented a greater efficiency against gram-positive bacteria, rather than gram-negative bacteria [41].

Inflammation represents a defensive, restoring process, with the major objective of protecting the organism. Causes of the inflammatory process may lie in the initial lesion, e.g., bacteria, fungi, parasites, toxins, etc., or in the consequences resulting from such said lesions, e.g., cellular and tissular necrosis [42]. The most important phenomenon consists in the permanent migration and recruitment of macrophages from within the capillary microcirculation. The regenerative tissular capacity is the result of two categories of factors. The first consists of the capacity of resting cells to reenter the division cycle. The second factor is represented by the efficient differentiation of stem cells from the lesioned tissue. The regeneration capacity of tissues and organs is specific to animal organisms. Many of the regeneration processes in mammals represent compensatory growth processes which imply cell hyperplasia and hypertrophy [43].

The effect of SE on plantar inflammation was modest, probably due to the low dose or the transitory effect of the phenolic compounds administered through the extracts. These findings are similar to previous reports in the literature, which attested the antioxidant and

anti-inflammatory activities of several *Trifolium* species determined by both in vitro and in vivo studies, employing various cell lines as well as mice models. These pharmacological properties are attributed to the genus' abundance of phenolic and isoflavonic compounds, as detailed in the previous subchapters of this study. These compounds have been reported to inhibit cyclooxygenase activity in human monocytes as well as macrophages [2,44–46]. In this sense, plant-derived natural compounds have been recognized as safe substances, with moderate effectiveness for the treatment of several inflammatory diseases [47]

5. Conclusions

A major observation was the tendency of extraction yields to increase proportionately along with the values of extraction parameters. However, this progression in yields halted abruptly once compound degradation temperatures were achieved, being also favored by other amplified conditions such as extraction time or speed. This phenomenon was most notable for TBE and UAE. One could conclude that the SE presented biological activity, considering the results of the phytochemical profile of the red clover extracts. Therefore, red clover could also constitute a medicinal herbal species with potential applications in complementary therapies, demonstrating safety and efficacy for antioxidant and antimicrobial activities.

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