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Green Extraction Techniques of Phytochemicals from *Hedera helix* L. and In Vitro Characterization of the Extracts

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Abstract: Phytochemicals from *Hedera helix* L. lead to extracts with good biological properties which are beneficial to human health and can be used to protect plants against different diseases. The aim of this research was to find the most suitable extraction method and the most favorable parameters for different bioactive compounds extraction from ivy leaves. Different extraction methods, namely microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), and conventional heating extraction (CHE), were used. The most suitable method for saponins extraction is MAE with an extraction efficiency of 58%, while for carbohydrates and polyphenols the best results were achieved by UAE with an extraction efficiency of 61.7% and 63.5%, respectively. The antioxidant activity (AA) of the extracts was also determined. The highest AA was obtained by UAE (368.98±9.01 μmol TR/gDM). Better results were achieved at 50 °C for 10 min of extraction, using 80% ethanol in water as solvent. In order to evaluate their in vitro cytotoxicity, the extracts richest in bioactive compounds were tested on NCTC fibroblasts. Their influence on the DNA content of RAW 264.7 murine macrophages was also tested. Until 200 μg/mL, the extracts obtained by UAE and MAE were cytocompatible with NCTC fibroblasts at 48 h of treatment.

Keywords: microwave; ultrasound; saponins; carbohydrates; polyphenols; antioxidant activity; in vitro cytotoxicity

1. Introduction

Common ivy (*Hedera helix* L.) is a medicinal plant which belongs to Araliaceae family and is native to Asia, North America, and Central, Western and Southern Europe [1]. Although common ivy is an inedible plant for both humans and animals, its leaves are rich in bioactive compounds with beneficial effects on the human body. In addition, botanical extracts rich in such phytochemicals can be used as biostimulants in horticulture or agriculture fields [2]. Regarding the ivy leaves, among these compounds, saponins are the most important. The structure of saponins implies the presence of at least one glycosidic bond at the carbon atom C3 between aglycone and a sugar moiety. Hydrolysis of saponins leads to an aglycone, usually named sapogenin, and a sugar moiety. *Hedera helix* mainly contains triterpenoid glycosides. The triterpenoids consist of three monoterpenes of 10 carbon atoms each, which are distributed as six isoprene molecules [3]. Among triterpene saponins, hederacoside C, hederagenin, and α-hederin are predominant in *Hedera helix* [4]. *Hedera helix* is also

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rich in polyphenols. These compounds contain one or more aromatic rings to which are attached one or more hydroxylic groups. In plants, they are usually linked by sugar moieties, being rarely found in their free form [5]. The polyphenols found in *Hedera helix* are caffeic and chlorogenic acids, kaempferol, rutin, and quercetin [6]. In *Hedera helix* leaves, there were also found monosaccharides with branched-chain, such as hamamelose and apiose and others sugars such as glucose, fructose, sucrose, and raffinose [7].

These bioactive compounds found in *Hedera helix* leaves provide to the extracts immunological [8], cytotoxic [9], antibacterial [10], antiviral [11], antifungal [12], anti-inflammatory [13], antiparasitic [14], antimutagenic [15], and antioxidant [16] properties.

The first step to use these bioactive compounds in industries, such as pharmaceutical, food, agricultural, cosmetic etc., is their extraction from *Hedera helix* leaves. The most common methods, such as heat reflux [17], maceration or Soxhlet [18] extractions, can imply long extraction times, high temperatures, excessive amounts of solvent (sometimes toxic solvents), and high energy consumption, which will result in low efficiencies and selectivity. Among non-conventional extractions, which can overcome these drawbacks are microwave and ultrasound assisted extractions. The microwave assisted extraction (MAE) process consists of heating both the solvent and plant tissue, thus improving the kinetics of extraction. During MAE, for temperatures above 45 °C, the plant material is heated selectively. Moreover, microwave heating targets the traces of water found in the cells of dried plant material and evaporation begins. By evaporation, the pressure created inside the cells increases. This will lead to pushing the cell walls from inside, producing elongation and eventually raptures. Thus, the temperature gradient created into the extraction mixture leads to a decrease in the chemical potential of the plant cell which will result in the cell wall rupture and, implicitly, to a facile release of the targeted component [19,20]. Ultrasounds consist of electromagnetic waves with frequencies from 20 kHz to 2000 kHz. When moving through a medium, alternate compression and rarefaction cycles of waves are involved. The propagation of acoustic waves in liquids produces the cavitation phenomenon described by the generation of microbubbles or cavities. These bubbles grow to a maximum size and collapse generating cavitation. The presence of a solid material near the collapsing bubbles can produce an asymmetric collapse. Thus, the cavitation can promote rupture of the cell wall and, therefore, leading to an increase in mass transfer rate between solvent and plant material, also increasing the permeability of cell walls [21].

We report here the evaluation and screening of the most suitable extraction method and the most favorable parameters for extraction of different bioactive compounds, such as saponins, carbohydrates, and polyphenols, found in *Hedera helix* leaves. The antioxidant activity of the extracts is also reported. Finally, in vitro analysis of the extracts richest in bioactive compounds were conducted in order to evaluate their cytotoxicity on NCTC normal fibroblast cells and the influence on the DNA content of RAW 264.7 murine macrophages. The aim of these tests was to evaluate the extracts as potential sources of bioactive compounds with therapeutic use. To the best of our knowledge, a comparison of these phytochemicals' extraction methods has not reported yet and the microwave assisted extraction of different bioactive compounds from *Hedera helix* leaves has been employed, for the first time, in this work. There is only one study which uses microwaves, but the researchers applied a microwave pre-treatment of the *Caulis hederae sinensis* leaves, the extraction of saponins being performed with supercritical fluids [22].

2. Results and Discussion

Different methods of phytochemical compounds extraction from plants and herbs lead to different extraction yields and efficiencies. An important factor to consider in the manufacture of bio-based products is the increase in the bioactive components content. In the scaling-up processes, low energy consumption and economical cost must be considered. This means that a low specific energy (consumed energy per amount of compound) is required which can be accomplished by a high extraction yield. According to literature [23,24] among other parameters, extraction time, temperature, and ethanol concentration in water can affect the content of extracted compounds. The aim of this work was to study the effect of these three parameters on different extraction methods of

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Hedera helix leaves and to compare the phytochemicals extraction content and antioxidant activity (AA) for the following extraction methods: conventional heating extraction (CHE), ultrasound assisted extraction (UAE), and microwave assisted extraction (MAE). The non-conventional techniques (MAE and UAE) used in this work present several benefits such as good control of the heating process, shorter extraction time, better extraction efficiency and selectivity, and reduced levels of required energy.

3.1. Multiple Successive Extractions

In order to assess the efficiency of bioactive compounds extraction an information about the total amount of extractable phytochemicals in Hedera helix leaves is required. Thus, discontinuous multiple successive extractions were performed using the best extraction conditions obtained for each extraction method. The phytochemicals content – total saponins content (TSC), total carbohydrates content (TCC), total phenolic content (TPC) – obtained by multiple successive extractions, for each method, is shown in table 1.

Table 1. The phytochemical content of the multiple successive extractions (extraction conditions: 10 min, 80% ethanol in water, 50 °C).

Number of successive extractions	TSC[1] (mg DE/gDM)	TCC[2] (mg GE/gDM)	TPC[3] (mg GAE/gDM)	AA[4] (µmol TR/gDM)
Conventional heating extraction (CHE)				
1st	64.50±0.64	184.04±6.28	26.07±1.35	279.01±5.20
2nd	34.98±0.64	58.52±2.01	7.48±0.29	110.57±3.68
3rd	13.01±0.05	27.08±1.23	5.23±0.30	59.99±0.53
4th	9.50±0.10	16.29±1.99	3.61±0.80	19.72±2.08
5th	3.33±0.19	8.12±1.99	1.24±0.10	11.85±1.21
6th	1.10±0.03	4.73±1.00	0.56±0.25	3.14±0.20
Total	126.42	298.78	44.19	
Efficiency (%)	94.59	82.31	96.12	
Ultrasound assisted extraction (UAE)				
1st	74.50±1.50	224.17±8.53	30.02±0.75	368.98±9.01
2nd	33.97±1.42	80.01±5.39	7.24±0.12	123.55±6.47
3rd	12.28±0.53	38.81±3.98	1.75±0.22	76.48±4.11
4th	9.53±1.24	13.69±3.25	0.54±0.10	29.95±2.14
5th	8.66±1.77	5.14±1.15	0.45±0.06	14.95±1.11
6th	3.66±0.35	1.18±0.24	0.17±0.03	7.56±0.59
Total	132.61	363	40.17	
Efficiency (%)	99.22	100	87.34	
Microwave assisted extraction (MAE)				
1st	77.64±1.79	200.24±4.01	28.29±1.36	305.70±2.36
2nd	20.42±2.21	69.75±3.02	9.93±0.07	90.56±2.47
3rd	13.93±0.17	34.52±1.92	4.73±0.31	70.25±2.27
4th	11.89±0.00	16.22±1.09	1.73±0.01	22.04±1.02
5th	8.03±1.02	8.19±0.46	1.01±0.06	11.51±0.73
6th	1.74±0.68	2.03±0.39	0.29±0.01	5.03±0.31
Total	133.65	330.95	45.98	
Efficiency (%)	100	91.17	100	

[1] TSC – total saponin content; [2] TCC – total carbohydrates content; [3] TPC – total phenolic content; [4] AA – antioxidant activity.

In Table 1, it can be noticed that the maximum amount of phytochemical compounds was achieved by non-conventional methods (e.g. TSCmax = 133.65 mg DE/gDM and TPCmax = 45.98 mg GAE/gDM by MAE and TCCmax = 363 mg GE/gDM by UAE). For all bioactive compounds, more than 73% are extracted in the first two extractions, for all methods used. TSC of first extract varied

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between 64.5 and 77.64 mg DE/gDM, TCC ranged from 298.78 to 363 mg GE/gDM, while TPC ranged from 27.66 to 45.98 mg GAE/gDM.

The most efficient extraction methods are the non-conventional ones (for saponins, MAE and UAE efficiencies are higher than 99%, for carbohydrates MAE and UAE efficiencies are higher than 91%, and for polyphenols, MAE and UAE efficiencies are higher than 87%).

When multiple extractions from the same raw material are involved, after each extraction, the plant (although initially dried) will swell up and saturate with the solvent despite centrifugation of extraction mixture. Thus, a higher number of extraction cycles may increase the plant material to solvent ratio if the solvent volume remains constant for each extraction. This could affect the extraction rate and lead to a waste of solvent without an improvement in the content of the extracted phytocomponents. In order to study the influence of various parameters, the extraction efficiencies were compared with the maximum extractable content obtained by the multiple successive extractions.

3.2. The Influence of Extraction Time on the Bioactive Compounds Content

The extraction time is an important parameter, which affects the content of extracted products. The amount of extracted bioactive phytochemicals increases in time until an equilibrium between the solubilized components by the solvent and the un-extracted constituents from the plant cells is reached [25]. The efficiencies of emerging extraction techniques (UAE and MAE) were compared with the conventional method. CHE, UAE, and MAE were performed at different extraction times (1, 5, 10, and 20 min). The other parameters, such as temperature (50 °C), solvent concentration (80% aqueous ethanol), and plant material to solvent ratio (1/20 w/v) were maintained constant. The influence of extraction time on the TSC, TCC, TPC, and AA was evaluated. Figure 1 shows the effect of extraction time on the total content of bioactive compounds and on the antioxidant activity.

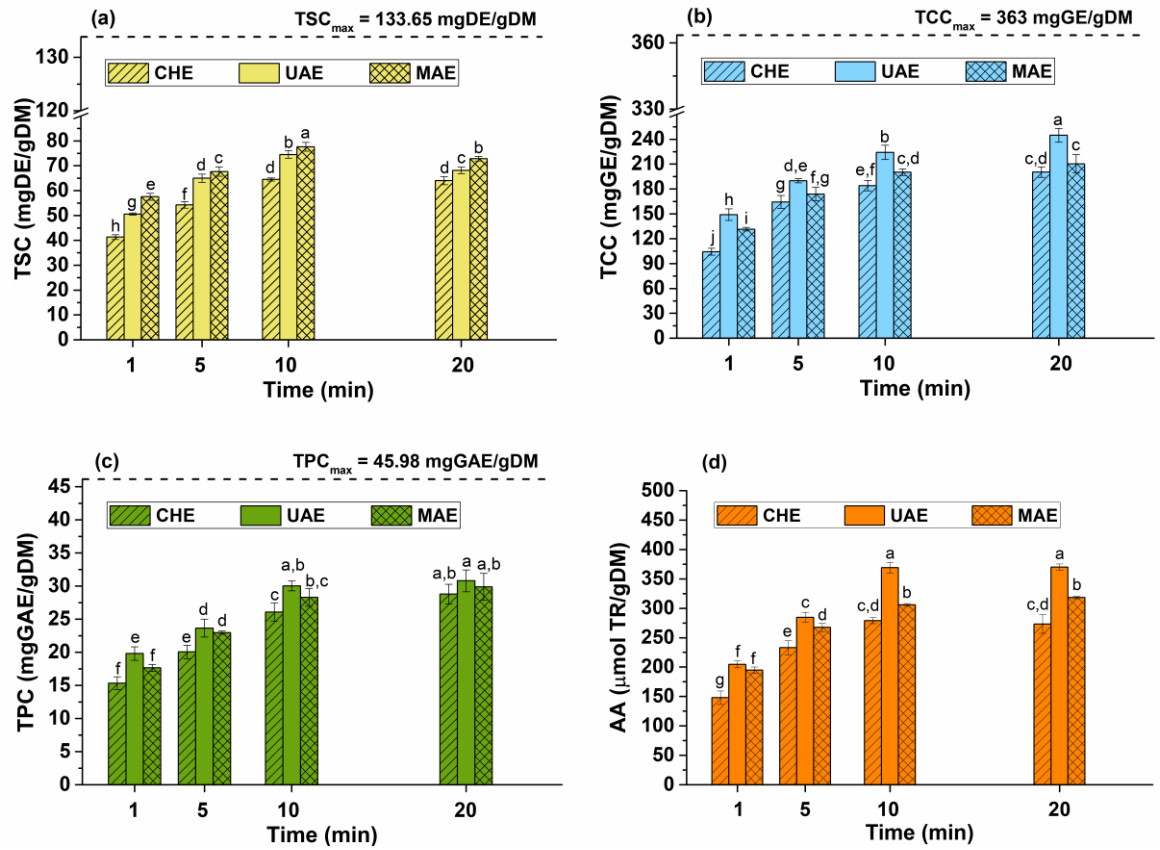

Figure 1. Influence of extraction time on the total content of bioactive compounds: (a) total saponins content (TSC), (b) total carbohydrates content (TCC), (c) total phenolic content (TPC), and (d) antioxidant activity (AA) – ivy leaves to solvent ratio of 1/20 (w/v), temperature of 50 °C, ethanol concentration in water of 80%, stirring rate of 900 rpm, ultrasound amplitude-40%. The significant

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difference between groups (p<0.05, ANOVA and Duncan's post hoc t-tests) are highlighted by different letters (a–j).

The results presented in Figure 1 reveal that for CHE, UAE, and MAE methods the total content of bioactive compounds increases with increasing the extraction time up to 10 min after which it decreases. The significant influence of extraction time on the total content of bioactive components and AA was confirmed by ANOVA and Duncan's post hoc t-tests. Considering the best extraction time 10 min, the results show the following:

- Saponins (Figure 2a): TSC increases in time until 10 min and after that, increasing the extraction time, a significant (p<0.05) decrease of extraction yield for all methods is observed. MAE led to the highest TSC (77.6 ± 1.7 mg DE/gDM), followed by UAE (74.5 ± 1.5 mg DE/gDM) and CHE (64.5 ± 0.6 mg DE/gDM).
- Carbohydrates (Figure 2b): TCC increases significantly in time until 20 min (p<0.05). UAE led to the highest TCC (224.2 ± 8.5 mg GE/gDM), followed by MAE (200.2 ± 4.0 mg GE/gDM) and CHE (184.0 ± 6.3 mg GE/gDM).
- Polyphenols (Figure 2c): TPC increases significantly in time until 10 min (p<0.05) with no significant differences by increasing the extraction time to 20 min. UAE led to the highest TPC (28.3 ± 1.2 mg GAE/gDM), followed by MAE (30.0 ± 0.7 mg GAE/gDM) and CHE (26.1 ± 1.3 mg GAE/gDM).
- Antioxidant activity (Figure 2d): AA is correlated with the phytocompounds content, the highest activity being achieved at 10 min. Extending the extraction time to 20 min, the AA increase was not significant. UAE led to the highest AA (368.9 ± 9.0 mmol TR/gDM), followed by MAE (305.7 ± 2.4 mmol TR/gDM) and CHE (279.0 ± 5.2 mmol TR/gDM).

According to Figure 1, the extraction yield increasing is not significant or tends to decrease within 10 and 20 min. Moreover, the extracted compounds might be susceptible to degradation due to a prolonged extraction time. Thus, the best extraction time is 10 min, when the highest amount of bioactive compounds was obtained.

3.3. The Influence of Temperature on the Bioactive Compounds Content

The extraction temperature is another important factor which may affect the solid-liquid extraction process. Due to limitations of the non-conventional methods, 40 and 50 °C were chosen to evaluate the influence of temperature on the content of bioactive compounds. Under 40 °C the microwaves are not effective and for temperatures higher than 50 °C some limitations can occur for UAE because it is known that the effect of ultrasounds is better at lower temperatures [26]. By increasing the temperature, bubbles can be formed in higher quantity and their collapse is less violent (due to the cushioning effect of vapors in bubble). Thus, the mass transfer enrichment caused by cavitation is reduced. In addition, when the temperature is too high, it might affect the molecular structure of phytocompounds, which will be destroyed, reducing the extraction yield implicitly. For these two temperatures the saponins, carbohydrates, and polyphenols were quantified and together with AA are presented in Figure 2.

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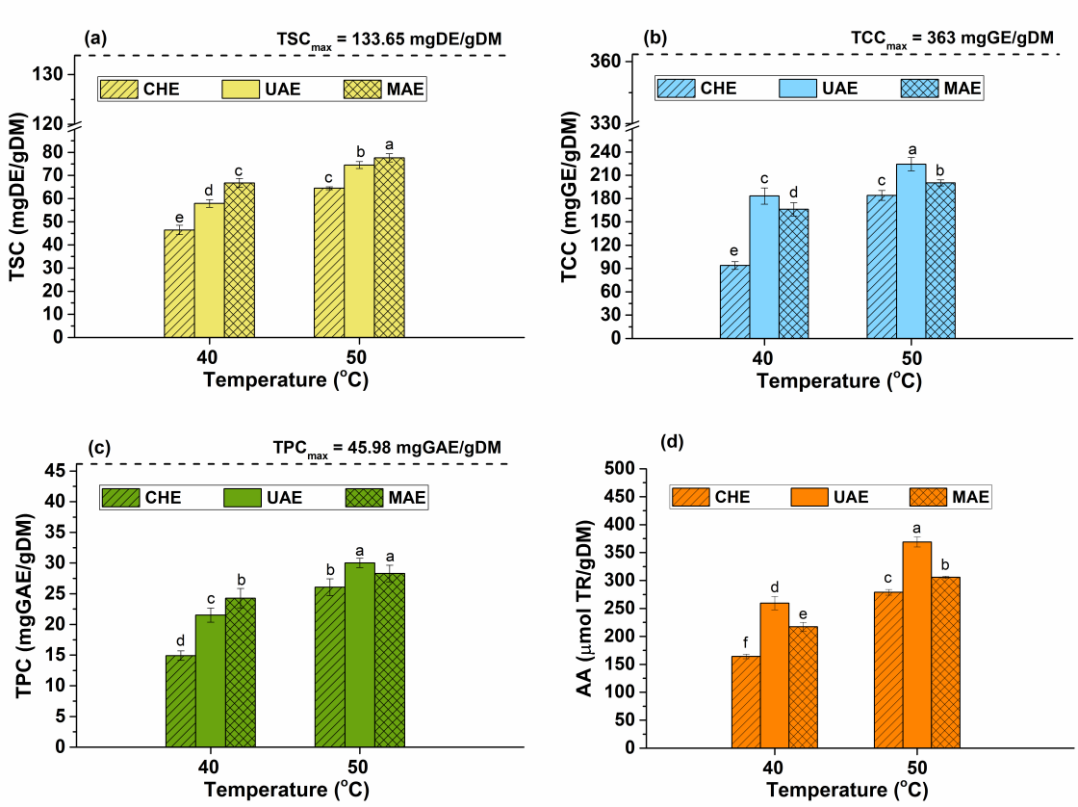

Figure 2. Influence of temperature on the total content of bioactive compounds: (a) TSC, (b) TCC, (c) TPC, and (d) AA – ivy leaves to solvent ratio of 1/20 (w/v), extraction time-10 min, ethanol concentration in water-80%, stirring rate-900 rpm, ultrasound amplitude-40%. Different letters (a–f) indicate the significant difference between studied groups (p<0.05, ANOVA and Duncan's post hoc t-tests).

As shown in Figure 2, for all bioactive compounds the highest amount was extracted at a temperature of 50 °C. Increasing temperature leads to an increase in solubility of bioactive compounds and enhances the mass transfer rate between solvent and vegetal material matrix. A high temperature can determine rapid ruptures of the cell wall and promote facile release of phytocompounds. However, a long extraction time and a high temperature may lead to the oxidation of bioactive compounds or may modify the conformation of extracted compounds. Moreover, in the case of thermolabile phytocompounds, maintaining the temperature at low values avoids the phytochemicals degradation [20].

The extraction efficiency of studied phytochemicals is higher than 55 % for the non-conventional methods. A significant influence of the extraction temperature on the total bioactive compounds content was showed by ANOVA test and Duncan's post hoc t-tests (see Figure 2). MAE technique led to the highest TSC (Figure 2a) with an efficiency of 58% (p>0.05). Using UAE method, the highest values for TCC (efficiency of 61.7%, Figure 2b), TPC (efficiency of 65.3%, Figure 2c), and AA (Figure 2d) were achieved.

3.4. The Influence of Solvent Concentration on the Bioactive Compounds Content

Another important parameter for phytocompounds extraction is the solvent concentration. In order to select the proper solvent for the extraction process, various properties such as viscosity, vapor pressure, surface tension, and solubility of components must be considered [27].

Ethanol is a green solvent widely used for the extraction of bioactive compounds from different plants due to its low toxicity. To improve the extraction efficiency, ethanol is commonly used at different concentrations in water. For this study different concentrations were used. The influence of solvent concentration on the phytoconstituents content is shown in Figure 3.

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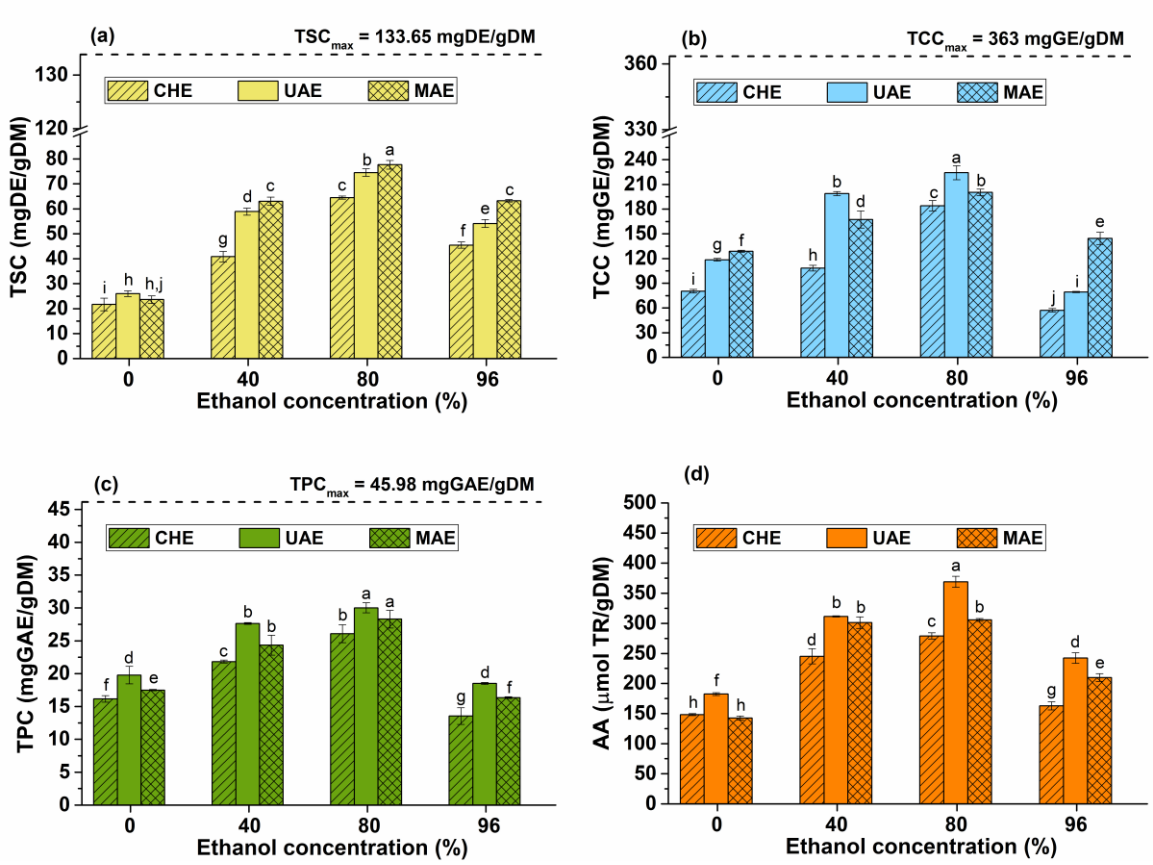

Figure 3. Influence of ethanol concentration on the total content of bioactive compounds: (a) TSC, (b) TCC, (c) TPC, and (d) AA – plant material to solvent ratio of 1/20 (w/v), extraction time of 10 min, temperature of 50 °C, stirring rate of 900 rpm, ultrasound amplitude of 40%. Different letters (a–j) express the significant difference between groups (p<0.05, ANOVA and Duncan's post hoc t-tests).

The total content of bioactive compounds increases with ethanol concentration up to 80% is reached. The significant influence of ethanol concentration on the extraction yield was confirmed by ANOVA test and Duncan's post hoc t-tests (Figure 3). Further increase in ethanol concentration does not enhance the yield of the target components.

As shown in Figure 3a, the highest TSC is obtained at an ethanol concentration of 80% for MAE. However, there are small differences between MAE and UAE, the recovery efficiencies being 58% and 55.7%, respectively. The saponins extraction with 96% ethanol led to a decrease in saponins content. This behavior can be explained by the polarity similarities between 80% ethanol and saponins, thus improving the extraction of these components [28].

The carbohydrates content increases with increasing the ethanol concentration up to 80% ethanol but decreases drastically at a concentration of 96% (see Figure 3b). This can be due to the lower solubility of carbohydrates in ethanol compared to aqueous ethanolic solutions. It can be noticed, in Figure 3b, that the carbohydrates content for 96% ethanol is lower than those extracted in distilled water. Comparing the extraction methods, the highest amount of carbohydrates was obtained by UAE with a 61.7% efficiency, followed by MAE with a 55.1% efficiency.

The polyphenols follow the same behavior as carbohydrates and saponins: the 80% ethanol concentration led to the highest TPC. The best results were achieved by UAE (65.3% efficiency), followed by MAE with 61.5% efficiency.

The AA of the extracts performed in 80% ethanol concentration was significantly higher than the one of the extracts carried out in distilled water or with 40 and 96% ethanol concentration (see Figure 3d.). As shown in Figures 3a-3c, the TSC, TCC, and TPC for the ethanol solutions ranged between 40 and 80% concentration were significantly higher than those obtained by 96% ethanol and distilled water. The UAE led to the highest AA, which is in concordance with the highest content of

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polyphenols, carbohydrates, and saponins. Consequently, the high content of bioactive components of the extracts performed in 80% ethanol led to a significant increase of AA. This was confirmed by ANOVA and Duncan's post hoc t-tests. Analyzing the data, it can be concluded that, for the extraction of phytoconstituents from *Hedera helix* leaves using different extraction techniques, the suitable ethanol concentration is 80%.

3.5. Principal Component Analysis

Principal Component Analysis (PCA) was performed in order to study possible correlations between TSC, TCC, TPC, AA and extraction methods (CHE, UAE, and MAE) of these compounds from *Hedera helix* leaves. For the multivariate analysis, the total phytochemical content and antioxidant capacity of the extracts obtained by different extraction techniques were determined. The bioactive compounds and antioxidant activity in the plane formed by the first two axes (PCs) explain 96.11% (eigenvalues >1) of the total variation, as well as 91.70% on the first axis and 4.41% on the second one. The coordinates of variables (factor loadings) on the factor-plane PC1−PC2 with the significant levels marked in bold are shown in Table 1S. The projections of cases (factor scores) on the factor-plane PC1−PC2 and the description of each method are summarized in Table 2S.

The PCA bi-plot and the correlation matrix are shown in Figure 4 and Table 3S, respectively. The significant values of correlation coefficients (r) are highlighted in bold at a significance level $\alpha = 0.05$ (two-tailed test).

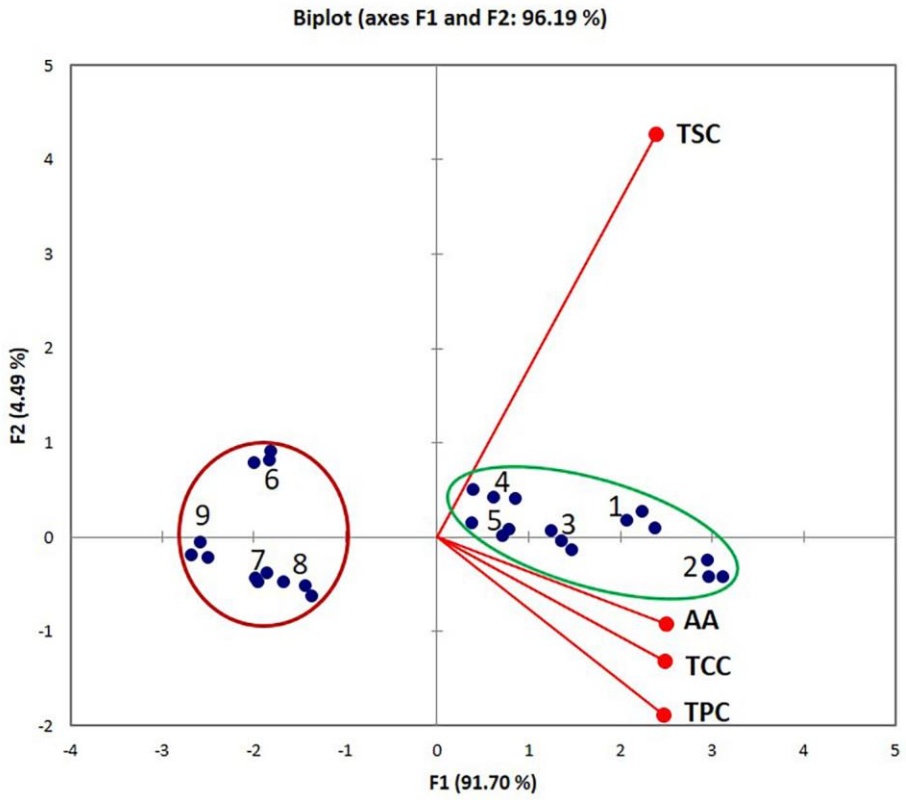

Figure 4. Projections of variables (TSC, TCC, TPC, and AA) and extraction methods (1–9) on the factor-plane PC1–PC2.

From the information of the factor loadings and scores presented in Tables 2S-4S and Figure 4, it is possible to correlate the extraction method and extraction conditions with the total bioactive compounds content and antioxidant capacity. Thus, the following observations were made:

- TCC, TPC, and AA show a positive influence on the PC1, and TSC shows a positive influence on PC2. Moreover, there is a high correlation between TCC, TPC, and AA. AA is highly positive

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correlated with TCC (r=0.928), TPC (r=0. 922) and positive correlated with TSC (r=0.867) – angles between vectors are lower than 90 degree;

- Based on PCA plot, two main groups of extraction samples were distinguished: group 1-5 (green ellipsoid) and group 6-9 (red circle). The extracts obtained by methods 1–5 (highlighted by green ellipsoid) had the higher content of bioactive components and the highest antioxidant activity compared with the extracts obtained by methods 6–9 (highlighted using red circle—discrimination on PC1).

3.6. Energy Considerations

During all experiments, the ultrasound and microwave powers were recorded. The power input for the heating plate, used for the conventional extraction, was measured using a Wattmeter. These recorded powers were used to determine the total energy introduced into the systems. Further, the specific energy was calculated using the following equation:

$$E_s = E_{total}/m_{Phc}\ [kJ/g\ of\ phytocompounds], \quad (1)$$

where Es is the specific energy [kJ/g of phytocompounds], E_{total} is the total energy introduced into the system [kJ], and m_{Phc} is the total amount of phytocompounds (the sum of saponins, carbohydrates, and polyphenols) obtained by each extraction method [g].

The total and specific energies for each method are presented in Table 2. The energy consumption for CHE is between 360 and 378 kJ, while for MAE and UAE range from 7.1 to 9.2 kJ and from 16.2 to 28 kJ, respectively. Although the lowest energy is consumed for the extraction at a temperature of 40 °C, for both UAE and MAE, the lowest specific energy is achieved for the experiments performed at a temperature of 50 °C using a concentration of 80% ethanol in water (see Table 2, lines 3 and 4). For these extraction conditions, the specific energy for CHE is 47 and 25 times higher compared with MAE and UAE, respectively. Regarding the ethanol concentration in water, it can be noticed in Table 2 that the best results are achieved for 80% ethanol. The energy considerations presented in Table 2 reveal that MAE is the greenest method, leading to the lowest specific energy, despite extraction conditions, when all phytocompounds from the same extract are considered (saponins, carbohydrates, and polyphenols). However, compared with CHE, both non-conventional methods lead to significantly less energy consumption, despite extraction conditions. Thus, in order to obtain extracts rich in bioactive compounds with strong antioxidant properties (such as saponins and polyphenols) both MAE and UAE procedures can be considered.

Table 2. Energy consumption during phytocompounds extraction (extraction time of 10 min).

Method Description	Total Energy (kJ)	Total phytocompounds extracted from ivy leaves (g/g DM)	Specific Energy (kJ/g of phytocompounds)
CHE, 50 °C, 80% Ethanol	378	0.2746	1376.5
MAE, 50 °C, 80% Ethanol	9.0	0.3062	29.5
UAE, 50 °C, 80% Ethanol	18.0	0.3287	54.9
CHE, 40 °C, 80% Ethanol	360	0.1553	2318.8
MAE, 40 °C, 80% Ethanol	7.1	0.2613	27.2
UAE, 40 °C, 80% Ethanol	16.2	0.2627	61.6
CHE, 50 °C, 0% Ethanol	378	0.1182	3196.8

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MAE, 50 °C, 0% Ethanol	9.2	0.1700	54.2
UAE, 50 °C, 0% Ethanol	28.0	0.1643	170.2
CHE, 50 °C, 40% Ethanol	378	0.1710	2210.1
MAE, 50 °C, 40% Ethanol	9.1	0.2548	35.7
UAE, 50 °C, 40% Ethanol	23.0	0.2853	80.6
CHE, 50 °C, 96% Ethanol	378	0.1162	3252.1
MAE, 50 °C, 96% Ethanol	8.2	0.2240	36.5
UAE, 50 °C, 96% Ethanol	17.4	0.1521	114.4

3.7. Biological Avtivity

The data obtained from the in vitro cytotoxicity evaluation using MTT spectrophotometric method quantifies the proportion of living cells in NCTC culture after 48 h of treatment with various concentrations of the tested extracts. Cell proliferation of NCTC fibroblasts values after 48 h of treatment, depending on concentration of the sample, are shown Figure 5.

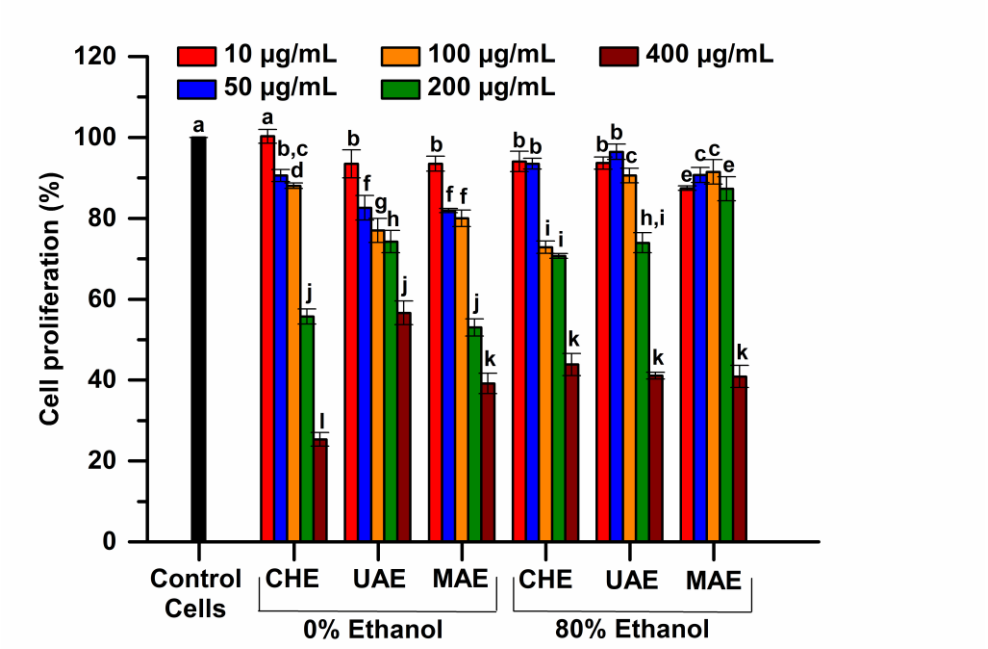

Figure 5. Cell proliferation of NCTC fibroblasts induced by the plant extracts at 48 h of treatment, determined by the MTT assay (extraction conditions of the selected extracts: temperature of 50 °C, extraction time of 10 min). The significant difference between groups (p<0.05, ANOVA and Duncan's post hoc t-tests) are highlighted by different letters (a–l).

The MTT assay at 48 h of treatment (for concentrations between 10 and 200 µg/mL) indicated that the extracts obtained by UAE and MAE methods with 80% ethanol manifested a high biocompatibility with NCTC fibroblasts, with cell proliferation values significantly higher (p<0.05) higher than 90%. On the contrary, the extracts obtained by CHE and MAE in 0% ethanol were cytotoxic, generating a low proliferation rate (lower than 56%). Following the obtained results about

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the interaction of plant extracts with NCTC fibroblasts, the correlation between tested concentration of the extracts and cell proliferation rate was observed.

A Live and Dead assay was performed to confirm the noncytotoxic activity of the plant extracts obtained by UAE and MAE in 80% ethanol, against RAW267.4 cell line. The results obtained after 48 h of treatment are shown in Figure 6.

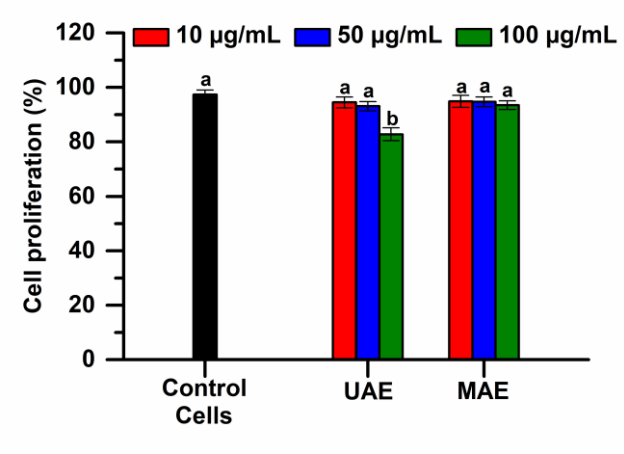

Figure 6. Cell viability of RAW 264.7 cells assessed by Live and Dead assay, after 48 h of treatment with the selected extracts (extraction conditions of the selected extracts: temperature of 50 °C, ethanol concentration in water of 80%, extraction time of 10 min). Letters (a,b) express the significant difference between groups (p<0.05, ANOVA and Duncan's post hoc t-tests).

This flow cytometry technique allowed the highlighting and discrimination of live cells from dead cells, the analysis showing that the cells cultured in the presence of the tested samples are positive for calcein-AM in a proportion of over 82% (p<0.05). These results indicate a good biocompatibility of the tested samples at 48 h of cultivation, at all concentrations tested.

3. Materials and Methods

2.1. Materials

Ivy leaves (*Hedera helix* L.) were collected in July 2022 at Hofigal S.A. With the help of an air flow-heating oven, the fresh ivy leaves were dried at 60 °C to constant mass. The final humidity was 5.8 %. The dried material was powdered and sieved to particles less than 315 µm in size. The dried and milled ivy leaves were dosed and sealed in plastic vessels samples of 25 g. The material was stored at 4 – 5 °C until it was employed for the extraction of phytochemicals.

The standards used for saponins, carbohydrates, polyphenols, and antioxidant activity determination were diosgenin, glucose, gallic acid, and Trolox, respectively, purchased from Sigma-Aldrich Co. The solvents ethanol and methanol, vanillin, sulphuric acid, phenol, Folin Ciocalteu reagent, sodium carbonate, cooper chloride, neocuproine, and ammonium acetate were of analytical grade and were purchased from Merck.

The in vitro experiments were performed using a NCTC (L929 clone) of mouse normal fibroblasts cell line and a RAW 267.4 murine macrophages cell line. Both cell lines were purchased from ECACC (Sigma-Aldrich) together with cell culture media of Minimum Essential Medium (MEM) and Roswell Park Memorial Institute 1640 Medium (RPMI). Fetal bovine serum (FBS) was produced by Biochrom, L-glutamins, and the antibiotics: penicillin, streptomycin and neomycin were purchased by Sigma Aldrich. The cytotoxicity assay of the extracts on NCTC cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT), purchased from Sigma Aldrich. For the assessment of cell viability by flow cytometry on RAW 267.4 cells, the LIVE/DEAD Viability Kit (Invitrogen) was used.

2.2. Bioactive Compounds Extraction Procedure

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Three extraction methods are compared in the present paper: conventional heating extraction (CHE), ultrasound assisted extraction (UAE), and microwave assisted extraction (MAE). The extractions of bioactive components were carried out in a batch system. The solvent used was a mixture of 0, 40, 80, and 96% ethanol in water. The plant material to solvent ratio was 1/20 (w/v). The extractions were performed at a temperature of 40 and 50 °C, for 1, 5, 10, and 20 min.

The UAE was performed in a special jacketed glass reactor using a Vibracell VCX750 (Sonics&Materials, Inc.; Newtown; USA) ultrasonic probe. The extraction temperature was kept at the proposed value by introducing water at convenient temperature into the extraction vessel jacket during all experiments. The sonication was applied in continuous mode, at an amplitude of 40%. The latter was chosen based on our previous work Gavrila et al. [4].

The MAE was carried out in a standard Biotage reactor using the microwave applicator Biotage®Initiator (Biotage Sweden AB; Uppsala; Sweden). The experiments were conducted at a stirring rate of 900 rpm. The latter was chosen based on our previous work Asofiei et al. [29].

In order to compare the efficiency of non-conventional extraction methods (UAE and MAE) of bioactive compounds from ivy leaves, experiments under CHE in the same conditions as non-conventional techniques were performed.

After extraction by each method, the mixture was centrifuged at 2500 rpm for 5 min, at room temperature. The supernatant was collected and freshly analyzed every time. All experiments were performed in triplicate.

2.3. Multiple Successive Extraction Procedure

The procedure of discontinuous multiple extraction implies adding a fresh portion of solvent over the same raw material after the mixture is centrifuged. This approach was repeated until the plant material was exhausted (six times), and each fraction collected was freshly analyzed. The discontinuous multiple extraction was performed in triplicate for all three methods.

The extraction efficiency of each phytochemical component was determined using the following equation:

$$E(\%) = \frac{Total\ amount\ obtained\ by\ each\ method}{Maximum\ extractable\ content} * 100 \quad (2)$$

2.4. Phytochemical Content Analysis

Phytochemical contents, such as saponins, carbohydrates, phenolics, and antioxidant activity were quantified using colorimetric methods. For all analyses, the absorbance was measured using a Shimadzu UV mini-1240 UV/Vis Scanning Spectrophotometer, 115 VAC (Shimadzu Deutschland GmbH; Duisburg; Germany). Each analysis was performed in triplicate for all extraction methods.

The total saponins content (TSC) was colorimetrically evaluated using the modified Hiai method [30], described in our previous work [4]. The absorbance was measured at 544 nm. The TSC was expressed as milligrams of diosgenin equivalents per 1 g of dry matter (mg DE/g DM) based on a standard curve corresponding to 40–550 mg/L diosgenin solution.

The total carbohydrates content (TCC) was determined colorimetrically using the method reported by Varkhade et al., with minor modifications [31]. Briefly, 1 mL of diluted extract, 1 mL of 5% phenol solution in water, and 5 ml of 98% sulfuric acid were added to a vial and mixed thoroughly, then let to rest at room temperature for 10 min. Further, the vial was placed in a water bath and maintained at 25-30 °C for 20 min, under constant stirring. The absorbance was measured at 490 nm. The quantification of TCC was presented as milligrams of glucose equivalents per 1 g of dry matter (mg GE/g DM) considering the standard curve which corresponds to 30–280 mg/L glucose solution.

The total phenolic content (TPC) was colorimetrically evaluated according to International Standard ISO 14502-1, with minor modifications. This determination is described in our previous work [32]. The absorbance was measured at 760 nm. The TPC quantification was given as milligrams of gallic acid equivalents per 1 g of dry matter (mg GAE/g DM) based on a standard curve corresponding to 1–5 mg/mL gallic acid solution.

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The antioxidant activity (AA) of the extracts was determined using the CUPRAC assay reported by Özyürek et al. [33], with minor modifications. Briefly, 1 mL of 0.01 M cooper (II) chloride aqueous solution, 1 mL of 0.0075 M neocuproine ethanolic solution, 1 mL of ammonium acetate buffer solution, and 1.1 mL of diluted extract were added to a vial and mixed thoroughly. Further, the mixture was kept in the dark, at room temperature, for 30 min. The absorbance was measured at 450 nm. The results were expressed as milligrams of Trolox equivalents per 1 g of dry matter (μmol TE/g DM). A standard curve which corresponds to 0–0.25 mg/mL Trolox solution was used for the AA quantification.

2.5. Cytotoxicity Assay

The biocompatibility of the plant extracts was evaluated on a stabilized NCTC mouse fibroblast cell line at 48 h of treatment using the cell viability MTT assay, carried out in accordance with the European standard SR EN ISO 10993-5:2009. The cells were seeded in culture plates with 96 wells at a cell density of 4x104 cells/mL in MEM culture medium with 10% fetal bovine serum and 1% antibiotics. They were incubated at 37 °C in a humid atmosphere with 5% CO_2, for 24 h. The experiment proceeded by putting in direct contact the cells from the wells with the solutions of plant extracts, at concentrations of 10, 50, 100, 200, and 400 μg/mL prepared in culture medium. At 48 h of treatment, the extracts in variable concentrations were replaced with a tetrazolium salt solution (5 mg/mL) and the experimental plate was incubated for 3 h under standard conditions, during which the specific reaction of the MTT assay generated formazan crystals in viable NCTC cells. After 3 h of incubation, the solution was removed from the wells and the formazan crystals were solubilized by adding isopropanol, resulting in a blue-violet coloring of the solutions from the wells. The spectrophotometric measurement of the color intensity of the solutions was carried out using a Berthold Mithras UV-Vis LB940 spectrophotometer (Germany), at a wavelength of 570 nm. The results were calculated as a percentage, based on untreated control cells, whose viability was considered 100%.

2.7. Live and Dead Staining

The assessment of cell viability of RAW 264.7 cells cultured for 48 h in the presence of the extracts was carried out by flow cytometry using the LIVE/DEAD Viability Kit, according to the manufacturer's recommendations (Invitrogen). For the analysis of cell viability, RAW 267.4 macrophages were seeded in 12-well culture plates, at a cell density of 1.5x105 cells/mL. The RAW 267.4 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin, streptomycin, and neomycin), being maintained at 37 °C, in a humid atmosphere with 5% CO_2. After 24 h, the cells were treated with samples in concentrations of 10, 50, and 100 μg/mL. After 48 h of cultivation in the presence of the samples, the cells were trypsinized and washed with phosphate buffered saline (PBS). Further, the cells were labeled with Live and Dead kit by incubation with calcein-AM and ethidium homodimer-1 for 20 min. The cells were then washed, resuspended in PBS and analyzed using the LSR II flow cytometer (Becton Dickinson) coupled with the FACSDiva software.

2.8. Statistical Analysis

All measurements were conducted in triplicate with the data expressed as mean value ± SD (standard deviation, n=3). All the results obtained at various levels of process factors were evaluated by univariate ANOVA and a multivariate principal component analysis (PCA) was performed. Duncan's new multiple comparison test at 95% confidence level was used for statistical analysis of the data, in order to detect the significant statistical differences between the averages of the main constituents of two or more independent groups. To estimate the power of linear correlations between dependent variables, the correlation Pearson coefficient (r) was used [34,35]. Statistical analysis was done with the help of XLSTAT Version 2019.1 (Addinsoft, New York, USA).

5. Conclusions

In this study, the most efficient extraction method of the active principles from ivy leaves in batch mode by CHE, UAE, and MAE was investigated. In order to determine the influence of different factors on the extraction process efficiency, a series of parameters were studied: extraction time, ethanol concentration in the extraction solvent, temperature, and multiple successive extraction. UAE and MAE have been shown to be an attractive alternative to the conventional method (CHE). The results indicated that the best parameters were an extraction time of 10 min, a temperature of 50 °C, and 80% ethanol as extraction solvent. For saponins extractions the highest value of TSC was obtained by MAE (77.64±1.79 mg DE/gDM). For carbohydrates and polyphenols, the best results were obtained by UAE (TCC = 224.17±8.53 mg GE/gDM and TPC= 30.02±0.75 mg GAE/gDM). PCA was performed to investigate possible correlations between TSC, TCC, TPC, AA and extraction methods (CHE, UAE, and MAE) of the bioactive compounds from *Hedera helix* leaves. A positive correlation between antioxidant activity and TSC (r=0.867), TCC (r=0. 928), and TPC (r=0. 922) was observed. The in vitro cytotoxicity experiment of ivy extracts assayed on NCTC normal fibroblasts showed a high degree of biocompatibility for the extracts obtained by UAE and MAE in 80% ethanol, at a concentration range from 10 to 200 μg/mL. The results were confirmed by Live and Dead flow cytometry assay performed on RAW 264.7 murine cells. On the contrary, the extract obtained by CHE in 0% ethanol, also tested in vitro on NCTC cells, manifested high cytotoxicity at concentrations higher than 200 μg/mL.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Table S1: Factor loadings; Table S2: Factor scores; Table S3: Correlation matrix.

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