**In Vitro Evaluation of Antidiabetic and Anti-Inflammatory Activities of Polyphenolic-Rich Extracts from Anchusa officinalis and Melilotus officinalis**

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**ABSTRACT:** This study was focused on the phytochemical composition and biological activities of Anchusa officinalis and Melilotus officinalis polyphenolic-rich extracts obtained by nanofiltration. The high-performance liquid chromatography–mass spectrometry analysis showed that chlorogenic acid and rosmarinic acid were the main phenolic acids in both extracts. The main flavonoid compound from A. officinalis extracts is luteolin, whereas rutin and isoquercitrin are the main flavonoids in M. officinalis. M. officinalis polyphenolic-rich extract had the highest α-amylase (from hog pancreas) inhibitory activity (IC$_{50}$ = 1.30 ± 0.06 μg/mL) and α-glucosidase (from Saccharomyces cerevisiae) inhibitory activity (IC$_{50}$ = 92.18 ± 1.92 μg/mL). However, both extracts presented a significant α-glucosidase inhibitory activity. Furthermore, the hyaluronidase inhibition of polyphenolic-rich extracts also proved to be stronger (IC$_{50}$ = 11.8 ± 0.1 μg/mL for M. officinalis and 36.5 ± 0.2 μg/mL for A. officinalis), but there was moderate or low lipoxygenase inhibition. The studies on the fibroblast cell line demonstrated that both A. officinalis and M. officinalis polyphenolic-rich extracts possess the cytoxic effect at a concentration higher than 500 μg/mL. The experimental data suggest that both extracts are promising candidates for the development of natural antidiabetic and anti-inflammatory food supplements.

1. **INTRODUCTION**

There is a growing interest in natural resources as a source for developing nutraceuticals, drugs, pharmaceuticals, and various cosmetics. However, different natural resources used in traditional medicine are not supported with sufficient scientific information about their chemical constituents and biological properties. In the recent years, many natural plant-based antioxidants either in the form of crude extract or functional foods are studied for their therapeutic potential in health management, such as diabetes or inflammatory processes. Scientific research supports the role of polyphenols in the prevention of diabetes mellitus and inflammatory processes. The bioactive compounds from extracts interact in a synergistic way, and it is supposed to be advantageous in chronic, multifactorial diseases involving multiple pathways.

Recent studies have shown that inflammation and oxidative stress are closely associated with diabetes, but the involved mechanism is not clearly established because of the dual role of oxidative stress as a signal and as a damaging agent (transcriptional control and cell cycle regulation). Application of the advanced technologies for the extraction of polyphenols and their concentration constitutes the first concern. Currently, ultrasonic-assisted extraction (UAE) or accelerated solvent extraction (ASE) and membrane technology are the effective techniques for extraction and "cold" concentration of the polyphenolic compounds from vegetable materials. Membrane technology has been used, especially for purification and concentration of added high-value compounds from fruit or vegetable juice, as a leading procedure in the field of separation technology in the past decades. Recent studies have recommended the nanofiltration process to be suitable for recovery of bioactive compounds from extracts. Microfiltration is used to reject a range of large-scale contaminants, such as suspended particles, major pathogens, bacteria, and colloids with a size range of 0.1–5 μm; therefore, it is used in the clarification and purification of water, juice, and vegetable extracts.

Anchusa officinalis (family: Boraginaceae) is a wild plant native to Europe, scarcely studied to date. The presence of...
polyphenols, pyrrolizine alkaloids, and triterpenoids in A. officinalis was confirmed in a recent research. Other species of the Anchusa genus, such as Anchusa italica and Anchusa strigosa, have a wide application in folk medicine, both exhibiting antimicrobial, antitumor, antiviral, anti-inflammatory, antidiabetic, and many other activities.

Melilotus officinalis known as yellow sweet clover (family: Fabaceae) is a widespread plant in Europe and Asia, but it also grows throughout the United States and Canada and has been used traditionally for anti-inflammatory and antiedematous activities, antiaging skin, and sprains and as a phlebotonic, spasmyloytic, diuretic, and sedative. The Melilot herb was mentioned in European Pharmacopoeia, the British Herbal Compendium, and Hagers Handbuch der Pharmazeutischen Praxis, which presented its pharmaceutical form and folk uses. Because of the high presence of coumarins, A. officinalis extracts have been tested clinically for the treatment of diabetic foot ulcers. Besides coumarin derivates, saponins, triterpenoid sapogenols, and flavones such as kaempferol, quercetin, rutin, and umbelliferone, the main compounds from this plant, other active principles are less known.

Despite a long tradition of use of these plants in medicine, their polyphenolic composition and antidiabetic effects are almost unknown. However, this study sought to examine the in vitro anti-inflammatory and antidiabetic activities of A. officinalis and M. officinalis polyphenolic-rich extracts.

2. RESULTS AND DISCUSSION

The effects of two modern extraction methods of polyphenolic compounds from aerial parts of A. officinalis and M. officinalis were investigated.

Both methods, UAE and ASE, are very useful technologies in the phytopharmaceutical extraction. In Table 1 are presented the extraction yields and polyphenol content obtained by each extraction technique. The extraction efficiency is estimated by the polyphenolic compound concentration. The highest extraction yields were reported by using the ASE technique, and the results disclosed that the ASE method was more effective for extraction of polyphenols (phenolic acids and flavonoids) from both herbal extracts compared to the UAE method. Zengin and collaborators evaluated the efficiency of extraction methods on polyphenols from Erica arborea L. extracts and obtained results similar to those presented in this study.

The high-performance liquid chromatography—mass spectrometry (HPLC–MS) analyses identified the presence of five phenolic acids, seven flavonoids, and one isoflavone (Table 2). Chlorogenic acid, rosmarinic acid, and luteolin were dominant in the extracts of A. officinalis, in accordance with another research, whereas isorhamnetin was identified in A. officinalis for the first time.

The obtained data revealed higher concentration for the most quantified polyphenolic compounds for the UAE method, which was opposite to the total phenolic and flavonoid content ASE > UAE (p < 0.05). The explanation could be the presence of other unidentified polyphenols in both the herbal extracts, which were extracted more efficiently by the ASE method. Our results are in agreement with previously published results such as the work by Nayak.

In order to further process crude extracts, large volumes of extracts are needed. In the case of ASE, the total time of extraction increases significantly; so to obtain 1000 mL of extract under the conditions mentioned in the method, the total extraction time was 255 min. Alternatively, the same volume of extract was obtained through the UAE method in 75 min. For this reason, the UAE crude extracts were used for purification and concentration by microfiltration and nanofiltration processes.

The most important attributes of UAE are the simplicity of technique, a short extraction time for a high volume of extract, and working at ambient temperatures, which prevent degradation of the thermolabile phenolic compounds.

Our results obtained in the present study (Table 3) are in agreement with previous studies that have shown a high efficiency for the concentration of polyphenolic compounds from the vegetable extract through nanofiltration, using a membrane with a molecular weight cutoff (MWCO) of 200–300 Da. A high concentration of polyphenols was also obtained by Brás and collaborators using a membrane with a MWCO of 400 Da.

As presented in Table 3, in total, 13 main polyphenolics were identified in both extracts. The chromatograms for the polyphenolic-rich extract are presented in Figures 1 and 2. Chlorogenic acid and rosmarinic acid were the main phenolic acid in both extracts, but some difference could be observed in the flavonoid composition. The main flavonoid compound from A. officinalis extracts is luteolin, whereas rutin and isoorquercitrin are the main flavonoids in M. officinalis.

This finding is in agreement with the presence of caffeic acid, luteolin, rutin, and isoorquercitrin reported by Liu and collaborators and Yang and collaborators in M. officinalis and also the presence of rosomicinic acid as the main phenolic acid in the ethanolic extract of A. officinalis reported by Boskovic and collaborators.

Evaluation of the antioxidant activity by the 2,2-diphenyl-1-picylhydrazyl (DPPH) radical scavenging assay (Table 4) proved that for all extracts, the nanofiltration retentates are more effective, but A. officinalis nanofiltrate retentate had the highest scavenging activity (IC50 = 0.0032 mg/mL), comparable with ascorbic acid used as the reference (IC50 = 0.0036 mg/mL), whereas M. officinalis extracts showed low DPPH inhibition.

Herbal medicines are an economical and efficient approach for the management of type 2 diabetes by inhibiting key enzymes related to this disorder. The α-amylase’s and α-glucosidase’s inhibitory activities of the crude extracts and concentrated extracts are shown in Table 4. M. officinalis crude and concentrated extracts showed a significant inhibitory activity against α-amylase enzyme when compared to the positive control acarbose. Furthermore, the concentrated extracts of A. officinalis and M. officinalis showed a significant α-glucosidase inhibitory activity, over 2.7 times better than that

| medicinal herb | extraction method | yield (%) | TPC, µg CAE/mL | TFC, µg RE/mL |
|----------------|------------------|-----------|---------------|---------------|
| A. officinalis | UAE | 17.0 ± 0.9 | 1589.37 ± 19.2 | 32.61 ± 2.4 |
| | ASE | 24.5 ± 1.8 | 2082.17 ± 28.5 | 83.04 ± 9.6 |
| M. officinalis | UAE | 21.1 ± 1.4 | 1879.52 ± 21.4 | 34.94 ± 2.8 |
| | ASE | 29.4 ± 2.1 | 2257.79 ± 26.1 | 67.64 ± 7.4 |

“TPC = total phenolic compound; TFC = total flavonoid content. Values represent means ± SD (n = 3).”
Table 2. HPLC−MS Polyphenolic Profile of the Extracts<sup>a</sup>

| compound [m/z] | UAE μg/mL | ASE μg/mL | UAE μg/mL | ASE μg/mL |
|---------------|-----------|-----------|-----------|-----------|
| chlorogenic acid [353] | 22.94 ± 2.4 | 10.48 ± 0.9 | 6.35 ± 0.6 | 8.03 ± 0.6 |
| caffeic acid [179] | 5.12 ± 0.3 | 1.72 ± 0.1 | 1.39 ± 0.1 | 1.17 ± 0.1 |
| rosmarinic acid [359] | 14.99 ± 1.2 | 8.39 ± 0.7 | 3.59 ± 0.2 | 2.10 ± 0.2 |
| ellagic acid [301] | – | – | 1.58 ± 0.1 | 0.24 ± 0.02 |
| p-coumaric acid [163] | 2.11 ± 0.2 | – | 1.61 ± 0.1 | 1.01 ± 0.1 |
| quercetol [301] | 0.12 ± 0.01 | 0.58 ± 0.05 | 0.73 ± 0.06 | 0.61 ± 0.04 |
| luteolin [269] | 30.22 ± 2.8 | 20.85 ± 1.8 | 1.40 ± 0.09 | 1.54 ± 0.1 |
| apigenin [269] | 3.04 ± 0.2 | 0.89 ± 0.08 | 0.50 ± 0.04 | 0.68 ± 0.06 |
| rutin [609] | 3.71 ± 0.3 | 8.26 ± 0.7 | 6.77 ± 0.5 | 6.05 ± 0.3 |
| quercetin-3-O-glucoside (isoquercitrin) [463] | 1.99 ± 0.1 | 5.59 ± 0.5 | 2.86 ± 0.2 | 2.50 ± 0.1 |
| kaempferol [285] | 0.17 ± 0.01 | 0.38 ± 0.03 | 0.34 ± 0.03 | 0.30 ± 0.02 |
| isorhamnetin [316] | 0.69 ± 0.05 | 0.55 ± 0.04 | – | – |
| genistin [433] | – | – | 1.49 ± 0.09 | 1.79 ± 0.1 |

<sup>a</sup>“−”, under limit of detection.

Table 3. HPLC−MS Polyphenolic Compounds of Extract Fractions<sup>a</sup>

| compound [m/z] | UAE extract µg/mL | retentate NF µg/mL | UAE µg/mL | retentate NF µg/mL |
|---------------|-------------------|-------------------|-----------|------------------|
| chlorogenic acid [353] | 22.94 ± 2.4 | 106.67 ± 8.4 | 6.35 ± 0.6 | 36.19 ± 2.4 |
| caffeic acid [179] | 5.12 ± 0.3 | 8.21 ± 0.5 | 1.39 ± 0.1 | 3.02 ± 0.2 |
| rosmarinic acid [359] | 14.99 ± 1.2 | 38.07 ± 3.2 | 3.59 ± 0.2 | 7.91 ± 0.6 |
| ellagic acid [301] | – | 1.58 ± 0.1 | 1.81 ± 0.1 | 1.64 ± 0.1 |
| p-coumaric acid [163] | 2.11 ± 0.2 | 3.76 ± 0.0 | 1.58 ± 0.1 | 1.81 ± 0.1 |
| quercetol [301] | 0.12 ± 0.01 | 0.18 ± 0.01 | 0.73 ± 0.06 | 1.24 ± 0.1 |
| luteolin [285] | 30.22 ± 2.8 | 3.76 ± 0.0 | 1.58 ± 0.1 | 1.81 ± 0.1 |
| apigenin [269] | 3.04 ± 0.2 | 4.65 ± 0.2 | 0.50 ± 0.04 | 0.74 ± 0.06 |
| rutin [609] | 3.71 ± 0.3 | 5.87 ± 0.4 | 6.77 ± 0.5 | 10.69 ± 0.9 |
| quercetin-3-O-glucoside (isoquercitrin) [463] | 1.99 ± 0.1 | 4.76 ± 0.3 | 2.86 ± 0.2 | 9.06 ± 0.8 |
| kaempferol [285] | 0.17 ± 0.01 | 0.23 ± 0.02 | 0.34 ± 0.03 | 0.48 ± 0.04 |
| isorhamnetin [316] | 0.69 ± 0.05 | 1.64 ± 0.1 | – | – |
| genistin [433] | – | – | 1.49 ± 0.09 | 4.22 ± 0.3 |

<sup>a</sup>“−”, under limit of detection.

Figure 1. Chromatograms of polyphenolic-rich extract A. officinalis UAE and ASE (inset, corresponding chromatograms) extracts obtained with the HPLC−MS method (1-chlorogenic acid peak, 2-p-coumaric acid peak, 3-rutin peak, 4-isoquercitrin peak, 5-rosmarinic acid peak, 6-quercetol peak, 7-luteolin peak, 8-apigenin peak, and 9-kaempferol peak).
of acarbose used as the standard. Our data revealed that the *M. officinalis* concentrated extract had the highest α-amylase (IC\textsubscript{50} = 1.30 ± 0.06 μg/mL) and α-glucosidase (IC\textsubscript{50} = 92.18 ± 1.92 μg/mL) inhibitory activities. The inhibitory activities of the main compounds identified in *A. officinalis* and *M. officinalis* extracts were higher than that of the therapeutic drug, acarbose (IC\textsubscript{50} = 17.68 ± 1.24 μg/mL for α-amylase and 272.58 ± 5.43 μg/mL for α-glucosidase). Among these compounds, rosmarinic acid and chlorogenic acid exhibited significant inhibitory activities against α-amylase and α-glucosidase (Table 4).

In the current study, the inhibitory effects of *M. officinalis* polyphenolic-rich extract on α-amylase and α-glucosidase were evaluated, and the results are similar to those described in the literature for other *Melilotus* species. 43 However, there was no information available in the literature about the α-amylase and α-glucosidase inhibition activities on *A. officinalis*.

The studies showed that regular consumption of vegetables and specific tea in diabetic patients can lead to improved glycemic control, enhanced antioxidant defense system, attenuated oxidative stress, and inflammatory markers. 44,45 Among the chemical constituents, polyphenols, as the main antioxidant compounds, have demonstrated favorable effect in the management of diabetes mellitus as they regulate carbohydrate metabolism and stimulate insulin secretion but also affect fat metabolism. 46,47 Lipoxigenases (LOXs) and hyaluronidases (HYAs) are significant factors for the inflammatory process. The concentrated herbal extracts showed a similar inhibition value for each enzyme (Table 5).

### Table 4. Antioxidant Activity and α-Amylase and α-Glucosidase Inhibitory Activities of Crude and Concentrated Extracts and Their Representative Compounds

| sample                  | DPPH (EC\textsubscript{50}, μg/mL) | α-amylase (IC\textsubscript{50}, μg/mL) | α-glucosidase (IC\textsubscript{50}, μg/mL) |
|-------------------------|------------------------------------|----------------------------------------|---------------------------------------------|
| *A. officinalis* crude extract | 0.141 ± 0.002^a                      | 954.16 ± 7.46^a                        | 151.76 ± 4.30^a                             |
| *A. officinalis* NF retentate | 0.0032 ± 0.0001^b                    | 283.75 ± 4.92^b                        | 99.15 ± 2.81^b                              |
| *M. officinalis* crude extract | 0.858 ± 0.12^c                       | 1.32 ± 0.08^d                          | 146.64 ± 3.64^c                             |
| *M. officinalis* NF retentate | 0.459 ± 0.04^d                       | 1.30 ± 0.06^d                          | 92.18 ± 1.92^d                              |
| acarbose                |                                     |                                        |                                              |
| ascorbic acid (vitamin C) | 0.0036 ± 0.0002                      |                                        |                                              |
| rosmarinic acid          | 0.92 ± 0.07                         | 4.31 ± 0.14                            |                                              |
| chlorogenic acid         | 1.84 ± 0.05                         | 3.76 ± 0.09                            |                                              |
| luteolin                 | 19.53 ± 1.12                        | 28.69 ± 0.26                           |                                              |
| rutin                    | 11.42 ± 0.62                        | 6.58 ± 0.12                            |                                              |
| isoquercitrin            | 9.65 ± 0.43                         | 7.82 ± 0.34                            |                                              |

“Different letters were used to indicate statistically significant differences between groups at *p* < 0.01.

### Table 5. Fraction Extracts and Their Representative Compounds Inhibitory Activity on LOX and HYA

|                        | LOX inhibition | HYA inhibition |
|------------------------|----------------|---------------|
| **Extracts**           |                |               |
| *A. officinalis* crude extract | 223.3 ± 2.3^a  | 44.6 ± 0.3^a  |
| *M. officinalis* crude extract | 201.8 ± 1.8^a  | 36.5 ± 0.2^a  |
| *M. officinalis* NF retentate | 94.8 ± 0.4^b   | 11.8 ± 0.1^b  |
| **Standards**          |                |               |
| ibuprofen              | 69.7 ± 0.3     | 13.7 ± 0.1    |
| rosmarinic acid        | 110.4 ± 0.7    | 24.3 ± 0.4    |
| chlorogenic acid       | 81.5 ± 3.2     | 162.4 ± 4.8   |
| luteolin               | 31.2 ± 0.7     | 5.4 ± 0.08    |
| rutin                  | 46.3 ± 1.4     | 9.2 ± 0.7     |
| isoquercitrin          | 98.4 ± 5.1     | 11.3 ± 0.9    |

^a*p* < 0.0001.  ^b*p* < 0.001 versus control (ibuprofen).

All herbal extracts showed high HYA inhibition (IC\textsubscript{50} = 36.5 ± 0.2 μg/mL for the *A. officinalis* concentrated extract and IC\textsubscript{50} = 11.8 ± 0.1 μg/mL for the *M. officinalis* concentrated extract) comparable with selected standards, but a moderate LOX inhibition (IC\textsubscript{50} = 94.8 ± 0.4 μg/mL for the *M. officinalis* concentrated extract and low LOX inhibition (IC\textsubscript{50} = 201.8 ± 1.8 μg/mL for the *A. officinalis* concentrated extract).

It was reported that LOX and HYA inhibitory activities are closely related to the radical scavenging activity, and also the...
anti-inflammatory activity of the concentrated extracts may be linked to the higher polyphenolic content, especially chlorogenic acid, rosmarinic acid, rutin, luteolin, and isoquercitrin.\textsuperscript{48−50} This is in agreement with our data presented in Table 5.

LOX is the key enzyme in the biosynthesis of leukotrienes (hydroperoxides) that play an important role in the pathophysiology of several inflammatory diseases. One of the ways of antioxidant action is the inhibition of lipid hydroperoxide formation in the course of enzymic peroxidation. This can limit the availability of lipidic substrate necessary for the catalytic cycle of LOX.

Our \textit{in vitro} study is in agreement with the previous study on rabbits which showed the anti-inflammatory activity of \textit{M. officinalis} related to reducing the activation of circulating phagocytes and lowered citrulline production.\textsuperscript{26}

This is the first report that investigated the \textit{in vitro} LOX and HYA activities of these plant extracts.

One of the important conditions in using medicinal plants for different diseases is their biocompatibility with normal cells. So, in this study, the biocompatibility was tested by cytotoxicity on the cell line of mouse fibroblast cells from the NCTC line, clone L929.

The studies performed on the polyphenolic-rich extracts of \textit{A. officinalis} after 24 h of incubation showed cell proliferation at concentrations of 50, 100, 250, and 500 μg/mL (cell viability is 104.55; 102.02; 101.18, and 99.1%, respectively), values that exceed that of the culture witness (Mc). At a concentration of 750 μg/mL was observed a slight cytotoxic effect (67.88% cell viability) (Figure 3a). \textit{M. officinalis} polyphenolic-rich extract is not cytotoxic at concentrations of 50, 100, 250, and 500 μg/mL (cell viability is 98.65; 98.40; 91.18, and 73.4%, respectively) after 24 h (Figure 3a).

After 48 h of incubation, polyphenolic-rich extract of \textit{A. officinalis} did not cause cytotoxicity in the concentration range of 50−250 μg/mL (cell viability is 94.18, 84.55, and 81.10%, respectively) and caused moderate cytotoxicity at 500 μg/mL (51.64% cell viability) (Figure 3b).

Polyphenolic-rich extract of \textit{M. officinalis} shows similar behavior after 48 h of incubation. It is not cytotoxic in the concentration range of 50−250 μg/mL (cell viability is 94.04, 80.08, and 66.88%, respectively), yet only moderately cytotoxic at 500 μg/mL (41.70% cell viability) and highly cytotoxic at 750 μg/mL (Figure 3b). For both polyphenolic-rich extracts, the concentrations above 500 μg/mL were cytotoxic for L929 cells, and the cell viability decreased strongly; the recorded values were significantly lower (\(p < 0.05\)) than the culture control.

Figure 4, showed that the cells maintained their normal phenotype in cultures treated with 50 and 100 μg/mL of \textit{A. officinalis} and \textit{M. officinalis} polyphenolic-rich extracts; the cells are uniform with monochrome cytoplasm, with cell density similar to the culture control, and so the extracts are not cytotoxic. For the \textit{A. officinalis} polyphenolic-rich extract, it can be observed that at 250 μg/mL concentration, the cell density decreased slightly, but the samples remain viable. At the concentration of 500 μg/mL, we can observe certain changes: several rounded cells and granular cytoplasm, possibly containing cellular debris. The cell density is lower compared to the culture control; the extract has a slight cytotoxic effect.

The NCTC cells treated with 250 μg/mL of the \textit{M. officinalis} polyphenolic-rich extract showed some modification: the cells are rounded and their density greatly diminished. At concentrations of 500 μg/mL, the extract is moderately cytotoxic, with rare and modified cells. At 750 μg/mL, the both extracts become highly cytotoxic.

Another study showed the cytotoxicity of \textit{A. officinalis} on different tumor cell lines.\textsuperscript{48}

This is the first report of cytotoxicity on fibroblast cells for both plants: \textit{A. officinalis} and \textit{M. officinalis}. This data allowed selecting the optimal range of noncytotoxic concentrations of each polyphenolic-rich extract (up to 250 μg/mL).

3. CONCLUSIONS

This is the first report about the determination of isorhamnetin in \textit{A. officinalis} and also the first study to evaluate the \textit{A. officinalis} polyphenolic-rich extracts potentially used in the management of diabetes mellitus. In addition, this study exhibits for the first time the LOX and HYA inhibitory activities of these plant extracts. Therefore, data presented in this section could be assumed as an original contribution to the literature.

Further studies might be led to investigate the mode of action of these extracts in interacting with the oxidative, antidiabetic, and inflammatory pathways in animal models.

These data bring new support for the traditional utilization of \textit{A. officinalis} and \textit{M. officinalis} and display that both polyphenolic-rich extracts could be used as antidiabetic and anti-inflammatory agents at concentrations below 250 μg/mL.

4. MATERIALS AND METHODS

4.1. Plant Material. \textit{A. officinalis} herb and \textit{M. officinalis} herb were collected from Romanian flora. The voucher
specimen no. 658184 (2005) for *A. officinalis* and voucher specimen no. 657579 (2004) for *M. officinalis* were preserved in the herbarium of the University of Cluj, Romania.

**4.2. Extraction Methods.**

**4.2.1. UAE.** Crushed dried leaves and flowers (100 g) were combined with 1000 mL of ethanol 50% (v/v) and shaken for 10–15 min. UAE was achieved using an ultrasound equipment (Elma Transsonic T 460), 1 h.

**4.2.2. ASE.** Extraction of aerial parts of herbs was accomplished using a Dionex ASE 350 system (Thermo Scientific, Waltham, MA, USA) equipped with an auto-sampler carousel and a collection tray. The sample (15 g) of ground dried herbs was mixed with diatomaceous earth (Thermo Scientific) in 100 mL of extraction cell. The following conditions were used for the extraction: solvent—50% ethanol, temperature—80 °C, pressure—1500 psi, static time—10 min, and static cycle—3.

**4.3. Membrane Concentration Processes.** The crude extracts prefiltered with a filter paper was microfiltered with the cellulose acetate membrane of 0.45 μm pore size (Millipore). The microfiltrate was processed by nanofiltration in order to obtain polyphenolic-rich extracts. The process was conducted at 8 bar of transmembrane pressure using the KOCH membrane laboratory unit with a membrane with cutoff 200–300 Da made of polyamide (NP90; Dow Filtmec, Sterlitech Company, USA).

**4.4. Chemical Analyses.**

**4.4.1. Total Phenolic and Flavonoid Contents.** The total phenolic content was determined using the Folin–Ciocalteu method as described by Lin,52 and the results were expressed as rutin equivalent (RE) mg/mL.

**4.4.2. HPLC–MS Analysis.** Phenolic compounds were analyzed using a HPLC–MS system, LCMS-2010 detector (liquid chromatography mass spectrometer). The electrospray ionization interface and column (C18 Kromasil 3.5, 2.1 × 100 mm) were integrated to the HPLC system. All solvents and standard compounds were acquired from Sigma-Aldrich (Germany) and Roth (Carl Roth GmbH, Germany). The HPLC–MS analysis of polyphenols was performed with conditions, standards, and solvents described by Albu et al.53 The concentration ranges for standards used in the HPLC–MS analyses were between 0.1 and 5 μg/mL for ellagic acid, quercetol, kaemferol, isorhamnetin, and genistin, 0.5–10 μg/mL for caffeic acid, p-coumaric acid, apigenin, rutin, and isoquercitrin, and 1–50 μg/mL for chlorogenic acid, rosmarinic acid, and luteolin.

**4.5. DPPH Radical Scavenging Activity.** The DPPH radical scavenging activity of the polyphenolic-rich extracts was conducted according to the modified method of Bondet.54 Different concentrations of sample extract or standard were mixed with DPPH methanolic solution, and the absorbance was measured at 519 nm.

**4.6. In Vitro Antidiabetic Assays.**

**4.6.1. Inhibition of α-Amylase Activity.** The α-amylase inhibition was measured according to Ranilla et al.55 with a slight modification, as an anterior reported article.56 The sample solution α-amylase from hog pancreas (0.5 mg/mL) was preincubated at 37 °C for 20 min. Then, starch solution (1%) was added, and incubation was achieved at the same temperature for 30 min. The reaction
was added with dinitrosalicylic acid reagent. The absorbance was measured at 540 nm.

4.6.2. α-Glucosidase Inhibition Assay. α-Glucosidase inhibitory activity was determined using the method described by McCue et al. A mixture containing 50 μL of sample solution, 125 μL of α-glucosidase from Saccharomyces cerevisiae (0.5 U/mL in phosphate buffer solution, pH 6.9), and 700 μL of phosphate buffer was preincubated at 37 °C for 15 min. After this time, 125 μL of 5 mM p-nitrophenyl glucopyranoside was added, and the mixture was held for 30 min at 37 °C. The reaction was stopped by adding 1000 μL of 0.2 M Na2CO3, and the absorbance was read at 405 nm.

For the positive control in antidiabetic assays, acarbose was used. The α-amylase and α-glucosidase inhibitory activities were expressed as a percentage of inhibition (IC50).

4.7. In Vitro Anti-inflammatory Assay. 4.7.1. LOX Inhibition. LOX is a relevant point in inflammatory processes. The LOX inhibitory activity was determined using a spectrophotometric assay. The method is based on increase of absorbance at 234 nm because of the formation of 13-hydroperoxyocta-decadienoic acid in the lipoxygenation reaction. The mixture containing LOX solution (2200 U/mL) and borate buffer 0.2 M (pH 9.0) or extract in borate buffer was incubated for 15 min; then, linoleic acid was added to the mixture, and the absorbance was read at 234 nm for 2 min. Based on the % inhibition, the IC50 values were calculated for A. officinalis and M. officinalis polyphenolic-rich extracts. Ibuprofen was used as the standard.

4.7.2. HYA Inhibitory Activity Evaluation. The inhibition of HYA was evaluated using the modified method reported by Sahasrabudhe and Dedhar. Bovine HYA (100 μL; 400 U/mL) in acetate buffer (0.1 M) and different concentrations of sample (50 μL) were incubated for 20 min at 37 °C; then, CaCl2 12.5 mM (100 μL) was incorporated, and the mixture was incubated at 37 °C for 20 min. The reaction was initiated by adding sodium hyaluronate (250 μL; 1.2 mg/mL) and was incubated at 37 °C for 40 min; sodium hydrosulphite solution 0.4 M (100 μL) and potassium borate 0.4 M (100 μL) were added to the reaction mixture and incubated for 3 min in water at 90 °C. 10% p-Dimethyl-aminoenzaldehyde (3000 μL) was added to the cool reaction mixture, and the absorbance was read at 585 nm after 20 min. Ibuprofen was used as the standard.

4.8. Cytotoxicity. The cytotoxicity of polyphenolic-rich extracts was accomplished with the cell line of mouse fibroblast (NCTC clone 929, from the European Collection of cell Culture—Sigma-Aldrich, SUA) using the MTT test, as described in our previous paper. This spectrophotometric assay is an indicator for cellular metabolic activity for detection of cell proliferation as it measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. The NCTC cells cultured under standard conditions (3.5 × 104 cells/mL density) after 24 h of incubation were changed with the medium containing various concentrations of plant extracts (50, 100, 250, 500, 750 μg/mL). The samples were placed in a 96-well plate with seeded cells and incubated for 24 and 48 h at 37 °C in 5% CO2 air atmosphere. The culture control was untreated cells, and the positive control was H2O2 (2 μL/mL). After incubation with MTT solution (3 h, 37 °C), the plates were shaken for 15 min, and the absorbance was measured at 570 nm on a microplate (Sunrise Tecan, Austria). All analyses were performed in triplicate.

After the addition of the extracts, the morphological changes of the NCTC stained with Giemsa were noticed after 48 h with a Zeiss AxioStar Plus microscope.

4.9. Statistical Analysis. All data are expressed as the mean ± standard deviation (n = 3) and analyzed using Microsoft Excel. Statistical analysis was performed using Student’s t-test, and the values were considered significant when p < 0.05.

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**Notes**

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