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Evaluation of the Antioxidant, and Antidiabetic Properties of Flavonoids and Isoflavonoids-Rich Extracts of *Medicago sativa* and *Solidago virgaurea*

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Evaluation of the Antioxidant, and Antidiabetic Properties of Flavonoids and Isoflavonoids-Rich Extracts of *Medicago sativa* and *Solidago virgaurea*

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Abstract: The present study evaluated the antioxidant, antidiabetic properties, and biocompatibility of *Medicago sativa* and *Solidago virgaurea* extracts enriched in flavonoid and isoflavonoid compounds. The extracts were obtained by accelerated solvent extraction and laser irradiation. Then, nanofiltration was used for the concentration of flavonoid and isoflavonoid compounds from extracts. The extracts were analyzed for antioxidant capacity using DPPH radical scavenging and reducing power methods, while the antidiabetic property was tested by α-amylase and α-glucosidase inhibition and *in vitro* on a murine insulinoma cell line (β-TC-6). *M. sativa* obtained by laser irradiation and concentrated by nanofiltration had the highest DPPH$^{\bullet}$ scavenging (IC_{50} = 105.2±1.1 and reducing power activities (IC_{50} = 40.98±0.2 μg/mL). *M. sativa* extracts had higher inhibition on α-amylase (IC_{50} = 23.9±1.2, respectively 26.8±1.1), while *S. virgaurea* had the highest α-glucosidase inhibition (9.3±0.9 μg/mL respectively 8.6±0.7 μg/mL). The results obtained after evaluating the antidiabetic *in vitro* activity showed that the treatment with *M. sativa* and *S. virgaurea* flavonoid- and isoflavonoid-rich extracts stimulated the insulin secretion of β-TC-6 cells, both under normal conditions as well as in hyperglycemic conditions. This paper argued that *M. sativa* and *S. virgaurea* flavonoid-rich and isoflavonoid-rich extracts could be an excellent natural source with promising antidiabetic potential.

Keywords: green extraction; antioxidant; antidiabetic; *Medicago sativa*; *Solidago virgaurea*; flavonoid-rich extract; isoflavonoid-rich extract

1. Introduction

Flavones and isoflavones are polyphenolic compounds of widespread interest in the nutritional and medicinal fields. These natural compounds are considered essential components due to their antioxidative, anti-inflammatory, antimicrobial, anti-mutagenic, estrogenic effects, and anticancer properties, combined with their ability to modulate critical cellular enzyme functions [1-3].

The interest in natural biologically active compounds comes from the recognition that they have very few side effects compared to synthetic compounds, and that research in recent decades has demonstrated the importance of the synergistic effect of bioactive compounds in a natural mixture.

Studies show that a good choice of extraction technique, solvents, and extraction conditions can favor the content of target bioactive compounds in the final extract, leading to increased efficacy of the final product. Although intensive investigations have been needed in recent decades, researchers are still seeking stable plant sources of natural antioxidants and highly efficient extraction technologies. At present, there is more interest in green and sustainable extraction methods [4-7]. Green methods offer the advantages of a shorter extraction time, higher selectivity, and lower organic solvent expenditure. Among green extraction methods ultrasound-assisted and, accelerated solvent extractions are high-performance techniques and have been intensively studied in the last time in different areas, including biology, and the pharmaceutical and food industries [8-11]. Recently, a new method, laser irradiation earned a great value in extractive technology, but there is only a study about

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this method [12]. Laser irradiation is used to intensify the heat process and biomass accumulation in the medium, modify the structure of macromolecules, and increase the quantity of bioactive compounds in the final extract (e.g., polysaccharides, proteins, polyphenolics, minerals, etc.).

Regarding the species-rich in isoflavonoids and flavonoids, *Medicago sativa* (lucerne; Fabaceae family) contains significant quantities known as phytoestrogens [13,14]. *M. sativa* is one of the most prevalent forage crops but also has a long tradition of use in folk medicine for central nervous and digestive system disorders and also for the cure of differing other ailments, including cancer disease [15-18]. However, only a few research studies have been directed at the antidiabetic potential of *M. sativa* [19,20].

Solidago virgaurea (goldenrod; Asteraceae family) is a medicinal plant used in popular medicine for the treatment of numerous diseases, especially as a urological agent in kidney and bladder inflammation [21,22]. According to the literature, its pharmacodynamic activity is attributable to the presence of biologically active compounds, especially flavonoids which are considered the most essential [14,23,24].

Since flavonoids are thermolabile compounds, high consideration was paid to the extraction and concentration of these compounds in the present study. In this context, the aim of the present study was to compare the accelerated solvent extraction and laser irradiation extraction, coupled with concentration by nanofiltration, on the antidiabetic and antimicrobial activities of the extracts enriched in isoflavones and flavones from the two medicinal plants.

2. Materials and Methods

2.1. Materials

Flavonoid and isoflavonoid compounds: rutin, quercitrin, quercetin 3-β-D-glucoside, quercetin, isorhamnetin, formononetin, genistein, naringenin, biochanin A, and vitexin were purchased from Sigma–Aldrich (Schnelldorf, Germany), daidzein was obtained from Fluka (Buchs, Switzerland), luteolin and kaempferol were purchased from Carl Roth (Karlsruhe, Germany); 2,2-difenil-1-picrilhidrazil (DPPH), potassium ferricyanide, sodium carbonate (Na_2CO_3), dinitrosalicylic acid (DNS), α-amylase from hog pancreas, α-glucosidase from *Saccharomyces cerevisiae*, and 4-nitrophenyl α-D-glucopyranoside (NPG) have been purchased from Sigma–Aldrich, and iron chloride was bought from Fluka. All other used reagents, methanol (Riedel-de Haen), and ethanol (Chemical Company) were of chromatographic or analytical purity; the ultra-pure water was obtained using the distillation apparatus from Evoqua Water Technologies (Pittsburgh, USA).

The medicinal plants were collected from Cluj county (Romania), and voucher specimens were stored in the Herbarium of Babes-Bolyai University from Cluj-Napoca (code: 868.786 for *Solidago virgaurea L.*; code: 622172 for *Medicago sativa*).

2.2. Extracts Preparation

Two green extraction methods were used to study bioactive compound extraction's influence: accelerated solvent extraction (ASE) and laser irradiation (LE).

2.2.1. ASE extraction

Accelerate solvent extraction of dried and grounded *M. sativa* and *S. virgaurea* was realized by Dionex ASE 350 System (Thermo Scientific, USA). Each cell (100 mL) equipped with a cellulose filter was filled with 15 g of dried plant and diatomaceous earth and the ASE conditions were set as: solvent – ethanol/water (50/50, v/v), temperature –60°C, static time – 10 min, number of cycles – 3. According to the ASE extracts volume, the concentration of the extracts was 9% (w/v).

2.2.2. Laser irradiation extraction

LE extraction was performed in the same conditions with ASE: 9 g of the dry plant (aerial parts) was mixed with 100 mL of ethanol/water (50/50, v/v), and extracted for 30 minutes assisted with laser

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radiation at a combined 1270 and 1550 nm. The LE extraction used a steel extractor provided with a lid with two windows through which laser irradiation was done (Figure 1).

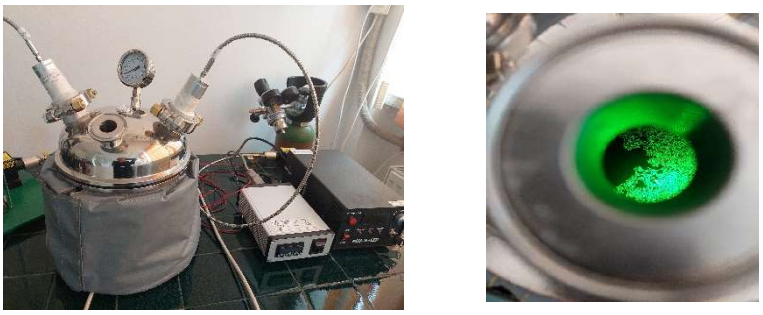

Figure 1. Laser-Assisted Extraction Installation.

Subsequently, all extracts were micro-filtrated through a Millipore membrane (0.45 µm pores) and concentrated by nanofiltration through Sterlitech membranes NF90 with a cut-off 150-300 Da using a KMS Laboratory Cell CF-1 module. The concentrated extracts were stored in a freezer at −20 °C for use in further analysis.

2.3. Analysis of flavonoids and isoflavonoids

2.3.1. Quantification of total flavonoids

Total flavonoid content was quantified using the aluminum chloride colorimetric method [25]. 2 mL of extract and 3 mL of methanol were mixed. After filtration, to 1 mL filtrate was added 1 mL sodium acetate solution, 0.6 mL of aluminum chloride solution, and 2.4 mL methanol. The absorbance was measured at 430 nm and the flavonoid content was calculated based on a rutin calibration curve ($y = 0.0073x - 0.0357$; $R^2 = 0.9959$).

2.3.2. HPLC-MS Analysis

HPLC analysis was realized using an HPLC Shimadzu system consisting of a SIL-20AC autosampler, two LC-20AD pumps, a DGU-20A degasser, and a CTO-20A column oven with an LC Solution software. The HPLC was coupled to a mass spectrometer detector, LCMS-2010 with an ESI interface using negative ionization mode and the following parameters: detector voltage, 1.8 kV; interface voltage 4 kV; heat block temperature, 200°C; CDL temperature, 200°C; interface temperature, 250°C and nebulization gas (N_2) flow rate, 1.5 L min^{-1}. A previously developed HPLC-MS method [26, 27] was used for the identification and quantification of polyphenol compounds, and analyses were performed on a Kromasil 100-5-C18 2.1x150 mm column and with an elution gradient of mobile phase (solvent A, formic acid in water, pH=3 and solvent B, formic acid in MeCN, pH=3) and a gradient of flow rate. The selected ion monitoring (SIM) mode was used and the corresponding peaks of the compound fragment ions ($[M-H]^-$: 163, 169, 179, 253, 267, 269, 271, 283, 285, 301, 315, 317 353, 431, 447, 463, and 609) were obtained for quantitative analysis.

2.4. Antioxidant Assays

2.4.1. DPPH Radical scavenging

The DPPH assay was carried out as described by Bondet et al. [28] with slight modifications. 100 µL extract with different concentrations was mixed with 1000 µL DPPH (2,2-diphenyl-1-picrylhydrazyl) 0.25 mM solution and 1.9 mL methanol. The absorbance was measured at 517 nm, and the extracts' scavenging activity was determined by the formula:

$$RSA\ (\%) = [(Ac - As)/Ac] \times 100, \quad (1)$$

where RSA = radical scavenging activity; Ac = control absorbance, and As = sample absorbance. Results were presented as inhibition, in EC_{50} (µg/mL). The values are reported as the mean ± SD.

2.4.2. Fe (III) Reducing Power Assay

Reducing power assay is based on the reduction of Iron (III) to Iron (II) and was performed using Berker's method [29]. The flavonoids and isoflavonoids-rich extracts (0.1 mL with varying concentrations) were mixed with 2.5 mL sodium phosphate buffer (0.2 M) and 2.5 mL potassium ferricyanide (1%) and then were kept at 50°C for 20 min. Thereafter, 2.5 mL of trichloroacetic acid (10%) was added. Finally, an aliquot of 2.5 mL mixture was combined with 2.5 mL water followed by 0.5 mL of iron chloride solution (0.1%) and UV absorbance was read at 700 nm. Results were presented as inhibition, in EC_{50} (µg/mL). The values are reported as the mean ± SD.

2.5. Antidiabetic Assay

2.5.1.α-. amylase and α-glucosidase inhibitory activities

The ability of extracts to inhibit α-amylase and α-glycosidase enzymes was examined to establish the plant's potential as an antidiabetic.

The α-amylase inhibition analysis was achieved according to our previous study [30]. Shortly, 100 µL of the extracts were to 250 µL α-amylase from hog pancreas (EC 3.2.1.1) solution in phosphate buffer (pH 6.9) and was maintained at 37°C for 20 min. Then, 250 µL starch solution was added and incubated at 37°C for 30 min. Subsequently, 500 µL DNS was added, and the mixture was heated at 90°C for 5 min. Absorbance measurements were performed at 540 nm.

The α-glucosidase inhibitory activity was evaluated using a slightly modified method of Ranilla et al. [31], with slight modifications. Samples, (60 µL) with different concentrations were incubated with 120 µL of α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20) solution (0.5 U/mL) and 720 µL phosphate buffer (0.1 M, pH 6.9), at 37°C, for 15 minutes. After that, 120 µL of NPG substrate solution was added, and the mixture was incubated at 37°C, for 15 minutes. Then, 480 µL of 0.2 M Na_2CO_3 solution was added to this mixture to stop the reaction, and the absorbance was read at 405 nm. The results were calculated using the formula:

$$\% \text{ amylase inhibition} = \frac{\Delta A_{control} - \Delta A_{sample}}{\Delta A_{control}} \times 100 \qquad (2)$$

Values were compared with the standard drug acarbose. IC_{50} values (concentration of the extract that inhibits 50% enzyme activity) were obtained from the nonlinear regression curve.

2.5.2. In vitro insulin secretion assay

In vitro, evaluation of antidiabetic activity was conducted on a mice insulinoma cell line (βTC-6). βTC-6 cells were purchased from Cell Lines Service (CLS, Germany) and were grown in DMEM medium supplemented with 10% FBS and 1% PSN antibiotic mixture at 37^0C and 5% CO_2.

βTC-6 cells were seeded in a 24-well plate, at a density of $1x10^5$ cells/mL. After 24 h of cultivation in standard conditions, βTC-6 cells were cultivated in normal (5.6 mM) and hyperglycemic (16.7 mM) conditions in the absence and presence of extracts for 1 hour, at 37°C. The culture medium was then collected, centrifuged for 10 minutes at 1500 rpm, and stored at -20°C until insulin measurement. Insulin secretion was determined by ELISA assay according to the manufacturer's recommendations (Sigma-Aldrich). L-alanine (10 mM) was used as the reference stimulant of insulin secretion from pancreatic beta cells.

2.6. Statistical Analysis

Three independent experiments were carried out and the obtained data were presented as mean ± standard deviation (SD) (n = 3). The sample pair of interest was analyzed using the paired

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Student's *t*-test (Microsoft Excel 2018 software). Significant statistical differences were considered $p<0.05$.

3. Results and Discussion

3.1. HPLC-MS analysis

As mentioned in the Introduction, one of the objectives of the study was to obtain the flavonoids and isoflavonoids-rich extracts of Medicago sativa and Solidago virgaurea using two green extraction methods: accelerated solvent extraction and laser irradiation extraction, coupled with concentration by nanofiltration.

The previous results of the authors demonstrated the efficiency of the nanofiltration process in the concentration of the processes of polyphenolic compounds (phenolic acids, flavonoids, isoflavonoids) [26, 32].

Accelerated solvent extraction (ASE) involves the use of solvents at high temperatures and pressures. High temperatures accelerate the kinetics of the extraction process, while increased pressure keeps the solvent below its boiling point, thus obtaining fast and safe extractions. However, taking into account the particularities of the compounds of interest, the use of high temperatures can cause their destructuring and loss of activity, the extraction was carried out in 3 extraction cycles at a temperature of 60°C.

The HPLC-MS method has been used for the flavonoid and isoflavonoid profile characterization of plant extract samples. The target bioactive compounds are presented in Table 1.

Table 1. Contents of target compounds in the extracts.

Compound	*M. sativa* flavonoid- and isoflavonoid-rich extract (µg/mL)		*S. virgaurea* flavonoid- and isoflavonoid-rich extract (mg/mL)	
	Conc. ASE	Conc. LE	Conc. ASE	Conc. LE
Rutin	157.16±9.6	58.48±4.8	2024.05±108.9	1652.05±99.7
Luteolin	10.43±1.2	8.43±0.7	2.13±0.2	2.88±0.2
Quercitrin	5.71±0.5	6.02±0.5	21.90±1.8	22.62±2.1
Quercetin 3-β-D-glucoside	23.04±2.2	73.07±6.2	175.14±11.6	183.11±12.9
Quercetin	1.10±0.1	10.69±0.9	2.48±0.1	17.09±1.4
Kaempferol	10.20±0.9	19.38±1.4	52.05±4.2	58.35±5.1
Isorhamnetin	1.29±0.1	0.47±0.04	6.52±0.5	3.59±0.3
Daidzein	2.47±0.2	1.65±0.1	-	-
Formononetin	4.13±0.3	2.72±0.2	0.55±0.04	0.30±0.02
Genistein	8.35±0.6	3.76±0.2	2.21±0.1	0.63±0.05
Naringenin	0.05±0.01	0.12±0.01	0.47±0.03	0.68±0.04
Biochanin A	0.25±0.02	0.36±0.02	0.61±0.03	0.67±0.03
Vitexin	7.00±0.5	65.30±4.9	126.58±8.9	158.15±11.8
Total	231.18±1.2	250.45±1.7	2414.69±10.5	2100.12±10.3

Conc.ASE – concentrated extract obtained after ASE extraction; Conc. LE – concentrated extract obtained after Laser extraction; Results are expressed as mean ± SD (n=3).

The data obtained showed that laser irradiation is a more efficient extraction method for some flavonoids and isoflavonoids (eg, quercetin 3-D glucoside, quercitrin, naringenin, and vitexin) than ASE extraction. Hence, this method that efficiently extracts these valuable compounds is of particular interest, especially since it has been very little studied. Lasser irradiation is a very new method of selective extraction, which demonstrated the efficiency in the extraction of polyphenols from plants at 552 nm, 660 nm, and 785 nm [12].

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Our studies were carried out with laser radiation at a combined 1270 and 1550 nm because we found that at wavelengths over 1200 nm the flavonoid compounds are extracted with much greater efficiency. This method was very efficient in the case of flavonoid and isoflavonoid compounds extraction from our studied plants, being able to obtain large amounts of extract, depending on the capacity of the extractor, with a lower time.

However, this is the first study that uses this combination of wavelengths in laser extraction, and that demonstrates the high efficiency in the extraction of flavonoids and isoflavonoids.

At the same time, by ASE, higher values were obtained for other compounds from the class of flavonoids and isoflavonoids. The comparison of the total flavonoid and isoflavonoid compound values in the samples indicates close values for the extracts obtained by both methods. Using a 50% (v/v) hydroalcoholic solution represents a reduction in the cost of the extraction process versus using pure solvents while maintaining a high extraction yield of the targeted compounds.

Several studies indicated that M. sativa is a rich source of phytoestrogens. HPLC-MS analysis showed that the highest rutin content was the dominant flavonoid from all plant extracts (Figures 2 and 3). Rutin, quercetin, kaempferol, naringenin, formononetin, and genistein were also reported in other studies [13, 14, 33]. However, biochanin A and vitexin were detected only in M. sativa seeds and sprouts not in the aerial part [34]. Among the isoflavones, vitexin is found in the largest quantity. The determined values of the phytoestrogens investigated in this research differed from the results obtained by the above-mentioned studies, which are most likely related to the type of cultivar, stages of maturity, extraction method, and other factors.

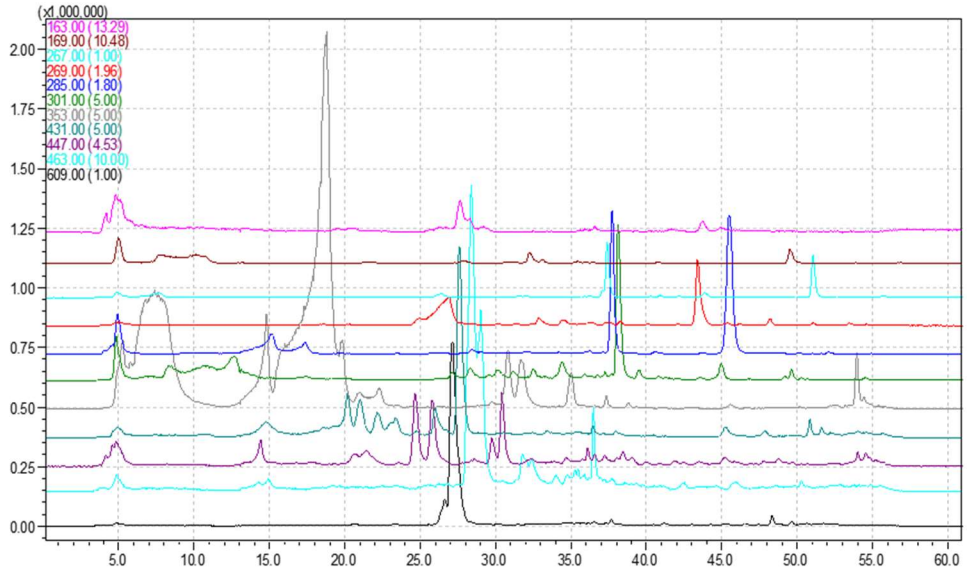

Figure 2. HPLC profile of flavonoids and isoflavonoids-rich extract of *M. sativa* ([M-H]:267-formonontin; [M-H]$^-$:269-genistein; [M-H]$^-$:285-luteolin and kaempferol; [M-H]$^-$:301-ellagic acid and quercetin; [M-H]$^-$:353-chlorogenic acid, [M-H]$^-$:431-vitexin; [M-H]$^-$:447-quercitrin; [M-H]$^-$:463-quercetin 3-β-D-glucoside; [M-H]$^-$:609-rutin) by HPLC-MS.

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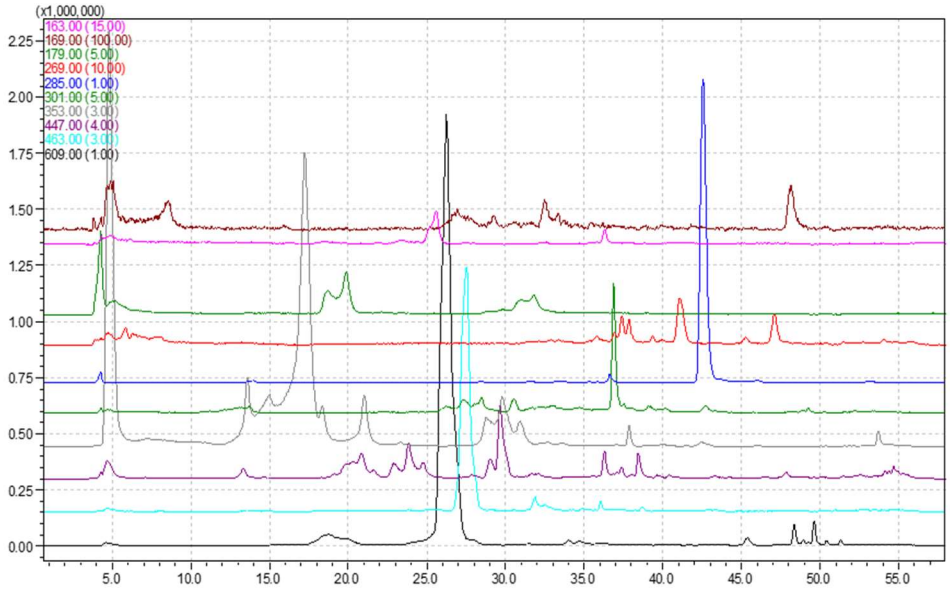

Figure 3. HPLC profile of flavonoids and isoflavonoids-rich extract of *S. virgaurea* ([M-H]:267-formononontin; [M-H]⁻:269-genistein; [M-H]⁻:285-luteolin and kaempferol; [M-H]⁻:301-ellagic acid and quercetin; [M-H]⁻:353-chlorogenic acid, [M-H]⁻:431-vitexin; [M-H]⁻:447-quercitrin; [M-H]⁻:463-quercetin 3-β-D-glucoside; [M-H]⁻:609-rutin) by HPLC-MS.

The HPLC–MS analysis of S. virgaurea extracts showed a significant content of rutin, quercetin 3-β-D-glucoside, and vitexin. Daidzein was not detected in S. virgaurea extracts. Our data confirmed those of previously published studies reporting significant amounts of rutin in S. virgaurea species [14,24]. To the best of our knowledge formononetin, biochanin A, and vitexin were not reported previously in goldenrod hydroalcoholic extracts.

3.2. Total Antioxidant activity

Flavonoids are a group of natural compounds with a biologically active potential; hence, they have an antioxidant effect. The results for the total flavonoid content and antioxidant activity (DPPH and Fe(III) reducing power methods) in the various extracts compared with ascorbic acid (vitamin C) as standard, known for its antioxidant properties are displayed in Table 2.

Table 2. Total flavonoid content and antioxidant activity of analyzed extracts.

Sample		Total flavonoid content, mg RE/mL	DPPH	Fe(III) reducing power
			IC_{50}, μg/mL	
M. sativa	**Conc. ASE**	**355.43±8.4**	278.7±2.5	42.29±0.3
	Conc. LE	426.70±11.2	105.2±1.1	40.98±0.2
S. virgaurea	Conc. ASE	1398.74±15.6	381.3±2.9	58.67±0.3
	Conc. LE	1382.56±13.8	198.4±1.6	56.92±0.4
Ascorbic acid			39.4±0.1	125±1.1

Conc.ASE – concentrated extract obtained after ASE extraction; Conc. LE – concentrated extract obtained after Laser extraction; RE: rutin equivalent. Results are expressed as mean ± SD (n=3).

The DPPH scavenging assay is a frequently utilized method to evaluate antioxidant activity. The IC_{50} values related to the DPPH radical scavenging activity for all extracts were higher than vitamin C showing a moderate antioxidant activity, *M. sativa* obtained by laser irradiation and concentrated by nanofiltration being the most active extract (IC_{50} = 105.2±1.1 μg/mL). The free radical inhibition results for *M. sativa* are in accord with the previous study which showed strong antioxidant activity

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of extracts obtained from *M. sativa* [35, 36]. Comparing the obtained results, we can observe that although the extracts of *S. virgaurea* have a much higher content of flavonoids, they have a lower antioxidant activity than *M. sativa*. This result can be explained by a higher content of other compounds with the antioxidant activity present in the extracts of *M. sativa* like phenolic acids or other isoflavone compounds not quantified in the studied extracts. Our results about the antioxidant activity of *S. virgaurea* extracts confirm the results of the other research, but it must be taken into account that the antioxidant activity is dependent on the solvent and the extraction method applied [37, 38].

The reducing power showed significant differences between the examined extracts compared to ascorbic acid as a standard, which showed the highest IC_{50}. *M. sativa* flavonoid- and isoflavonoid-rich extracts had the highest reducing power activity. The reducing power results revealed that all tested extracts had good abilities to donate electrons that were involved in the antioxidant activity.

3.3. Antidiabetic activity

α-amylase and α-glucosidase inhibition

One of the alternative approaches regarding the prevention/modulation of postprandial hyperglycemia is natural therapeutic inhibitors of α-amylase and α-glucosidase, as they are key enzymes in starch digestion. Our results for these enzymes' inhibition by the tested flavonoids and isoflavonoids-rich extracts are presented in Table 3.

Table 3. α-amylase and α-glucosidase enzymes inhibition of analyzed extracts.

Sample		α-amylase inhibition	α–glucosidase inhibition
		IC_{50} (μg/mL)	
M. sativa	**Conc. ASE**	23.9±1.2	24.2±0.9
	Conc. LE	26.8±1.1	25.7±1.1
S. virgaurea	Conc. ASE	33.9±2.4	9.3±0.9
	Conc. LE	32.1±1.9	8.7±0.6
Acarbose		24.2±1.6	66.5±4.2
Rutin		18.2±2.4	8.6±0.7

Conc.ASE – concentrated extract obtained after ASE extraction; Conc. LE – concentrated extract obtained after Laser extraction.

M. sativa extracts had higher inhibitory activity on α-amylase (IC_{50} = 23.9±1.2, respectively 26.8±1.1), while *S.virgaurea* had the highest α-glucosidase inhibition compared with acarbose, used as standard. *S.virgaurea* extracts showed the best α-glucosidase inhibition (IC_{50} of 9.3±0.9 μg/mL respectively 8.6±0.7 μg/mL) with almost 7 times lower than acarbose (IC_{50} of 66.5±4.2 μg/mL). The inhibitory activities of the rutin, the main compound identified in extracts were higher than those of the acarbose and it can be considered one of the compounds responsible for the activity of the extracts. The milder inhibition of α-amylase than α-glucosidase of all studied extracts could eliminate the major drawback of current drugs with side effects [39].

Flavonoids, such as rutin, quercitrin, and isoquercitrin (quercetin 3-β-D-glucoside) present in large quantities in the extracts from the present study, but also isoflavones (daidzein, genistein, vitexin) have been previously reported to have a hypoglycemic effect and stronger inhibitory effect on α-glucosidase [40-42]. This study suggests that combinations of flavonoid and isoflavonoid compounds from the studied plants have a synergic effect on α-amylase and α-glucosidase inhibition.

Effect of extracts on insulin secretion by β-TC6 cell lines

In this study, we investigated complementary *in vitro* the antidiabetic activity of *M. sativa* and *S. virgaurea* flavonoid-rich and isoflavonoids-rich extracts, at the tested concentrations (10-250 μg/mL), on a murine insulinoma cell line (β-TC-6).

The obtained results showed that higher insulin concentrations were obtained after treatment with all the tested extracts compared to the control, both in normal conditions (5.6 mM) and in hyperglycemic conditions (16.7 mM) (Figure 4).

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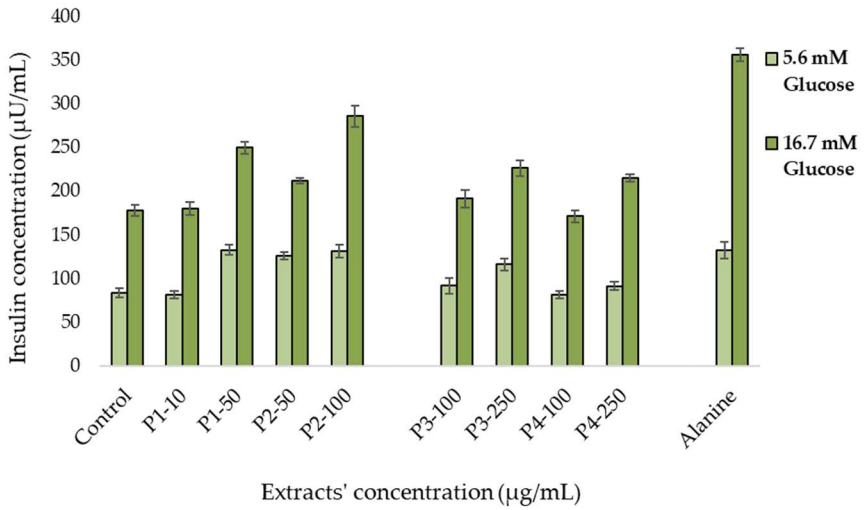

Figure 4. Effect of flavonoids and isoflavonoids-rich extracts on insulin secretion**.**

P1 – *S. virgaurea* concentrated extract obtained after ASE extraction; P2 - *S. virgaurea* concentrated extract obtained after LE extraction; P3 – *M. sativa* concentrated extract obtained after ASE extraction; P4 – *M. sativa* concentrated extract obtained after LE extraction.

In normal glycemic conditions (5.6 mM), the highest concentration of secreted insulin was obtained after treatment with *S. virgaurea* concentrated extract obtained after ASE extraction at 50 µg/mL (~134 µU/mL) and *S. virgaurea* concentrated extract obtained after LE extraction 100 µg/mL (~132 µU/mL). In the case of the control stimulated with 5.6 mM glucose and untreated, the secreted insulin concentration was ~84 µU/mL.

Likewise, similar results of the effectiveness of stimulating insulin secretion were also obtained in hyperglycemic conditions (16.7 mM). Thus, the best results were obtained after the treatment with *S. virgaurea* concentrated extract obtained after ASE extraction at 50 µg/mL (~249 µU/mL) and *S. virgaurea* concentrated extract obtained after LE extraction at 100 µg/mL (~286 µU/mL) and respectively *M. sativa* extracts 250 µg/mL (~226 µU/mL, and 215 µU/mL, respectively), for the stimulated and untreated control the secreted insulin concentration was ~178 µU/mL. Alanine was used as a positive control, demonstrating its ability to significantly stimulate insulin secretion.

Recent *in vivo* studies showed the anti-hyperglycemic effect of *S. virgaurea*, but the antidiabetic activity of *S. virgaurea* has rarely been studied [43,44]. However, the α-amylase and α-glucosidase inhibition by *S.virgaurea* extract wasn't found in the literature.

Interestingly, even though *M. sativa*, a related and well-known phytotherapeutic plant, is traditionally used as an anti-diabetic agent, its α-glucosidase- and α-amylase-inhibitory properties were very few investigated [45].

The correlation analysis for the bioactivity of flavonoids and isoflavonoids-rich extracts presented in Table 4 showed positive and negative correlations.

Table 4. Correlation coefficients between assays for flavonoids- and isoflavonoids-rich extracts.

	Pearson correlation coefficient (r)	**Significance ($p < 0.05$)**
	TFC	TFC
DPPH	0.758	0.04657
RP	0.877	0.02995
α-AMYL	0.967	0.02871
α-GLUC	-0.976	0.02925

TFC: total flavonoids content; DPPH, IC_{50}; RP: reducing power, IC_{50}; α-AMYL: α-amylase inhibition, IC_{50}; α-GLUC: α-glucosidase inhibition, IC_{50}.

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The correlation of DPPH radical scavenging ability, and reducing power with total flavonoid content remained significant (r = 0.758 and 0.877, $p < 0.05$), which indicated that flavonoid compounds play an important role in the antioxidant activity of *M. sativa* and *S. virgaurea* extracts. A strong and significant relationship was also observed between α-amylase and α-glucosidase inhibitory ability and total flavonoid content (r = 0.967 and -0.976, $p < 0.05$). This might be assigned to the flavonoid and isoflavonoid compounds under detection in HPLC-MS analysis.

4. Conclusions

In this research, the flavonoid and isoflavonoid profile, antioxidant, in vitro antidiabetic, and cytotoxicity of *Medicago sativa* and *Solidago virgaurea* extracts obtained using accelerated solvent extraction and laser irradiation extraction, coupled with concentration by nanofiltration were studied. The laser irradiation method at 1270 and 1550 nm combined wavelengths was very efficient in the case of flavonoid and isoflavonoid compounds extraction from *M. sativa* and *S. virgaurea* and was investigated for the first time. The extracts obtained by laser irradiation and concentrated by nanofiltration had the highest antioxidant activity. *M. sativa* flavonoid- and isoflavonoid-rich extracts had the best values for antioxidant activity (DPPH and Fe(III) reducing power methods). The flavonoid and isoflavonoid-rich extracts from both plants showed a significative inhibition on α-amylase and α-glucosidase, correlating with their total flavonoid and isoflavonoid high contents. Adittionaly, the obtained results showed that the studied extracts stimulate insulin secretion in vitro. S. viragurea flavonoid- and isoflavonoid-rich extracts showed the strongest stimulatory effect of insulin secretion in the in vitro β-TC-6 pancreatic beta cell stimulation model. Our results revealed that the *M. sativa* and *S. virgaurea* enriched with flavonoids and isoflavonoids could be used as an alternative therapy in the management of diabetes.

Author Contributions: G. Paun conducted research; G. Paun and E. Neagu obtained, processed, and analyzed the extracts; C. Albu was implicated in the HPLC-MS characterization; A. Alecu contributed to the laser irradiation extraction; A.M. Seciu-Grama anti-diabetic investigation; G. Paun and G.L. Radu made the final drafting work. All the authors have read and approved the final manuscript. The writing was realized by G. Paun.

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